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MODERN METHODOLOGIES

Edited by **MICHEL TIBAYRENC**

ENCYCLOPEDIA OF INFECTIOUS DISEASES

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Modern Methodologies

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Michel Tibayrenc

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Contributors

Mark Achtman, PhD, is a research group leader at the Max-planck-Institut für Inkektionsbiologie, Berlin, Germany. Research field: phylogenetic structure and ancestry of bacterial species.
Max-Planck Institut für Infektionsbiologie, Schumannstr. 21/22, 10117 Berlin, Germany
Phone: +49 30 28460751; fax: +49 30 28460750; email: achtman@mpiib-berlin.mpg.de; Web site: http://web.mpiib-berlin.mpg.de/mlst/AGroup

Shelley Adamo, PhD, works on the interactions between immune responses and behavior in insects. She is Professor at the Psychology Department at Dalhousie University Halifax.
Department of Psychology/Neuroscience Institute, Dalhousie University, Life Sciences Centre, 1355 Oxford St., Halifax, NS B3H 4J1, Canada
Phone: +1 902 494-8853; fax: +1 902 494-6585; email: sadamo@dal.ca; Web site: http://myweb.dal.ca/sadamo/lab4.1.htm

Arthur Aufderheide, MD, is Professor of Pathology and Director of Paleobiology Laboratory at University of Minnesota Duluth Medical School with research interest of Soft Tissue Paleopathology.
Department of Pathology, UMD Medical School, Duluth, Minnesota, 55812, USA
Phone: +1-218-726-7911; fax: +1-218-726-7559; email: aaufderh@d.umn.edu

Philip Awadalla, PhD, born 1969, has worked on population and evolutionary genetics theory and applications in model systems and disease. Is presently Assistant Professor of Genetics and the Bioinformatics Research Center at North Carolina State University.
Department of Genetics, North Caroling State University, Raleigh, NC 27695-7614, USA.
Phone: +1-919-513-7680; fax: +1-919-515-3355; email: pawadalla@ncsu.edu; Web site: http://www.cals.ncsu.edu/genetics/awadalla/awadalla.html

Francisco J. Ayala is University Professor and Donald Bren Professor of Biological Sciences at the University of California, Irvine. On June 12, 2002, President George W. Bush awarded him the National Medal of Science at the White House. Born in Madrid, Spain, he has lived in the United States since 1961, and became a U.S. citizen in 1971. He has published more than 900 articles and is author or editor of 30 books. His scientific research focuses on population and evolutionary genetics, including the origin of species, genetic diversity of populations, the origin of malaria, the population structure of parasitic protozoa, and the molecular clock of evolution.
Department of Ecology and Evolutionary Biology, University of California, Irvine, 321 Steinhaus Hall, Irvine, CA 92697-2525, USA
Phone: +1-949-824-8293; fax: +1-949-824-2474; email: fjayala@uci.edu; Web site: http://www.faculty.uci.edu/profile.cfm?faculty_id=2134

François Baillon, born 1952, is ornithologist and specialist of migrations between Europe and Africa, expert for use of Argos tags for the follow-up of bird fauna. He presently works on four species of migratory transsaharian prey birds and one stork. He is involved in the European program on the role of birds in the transmission cycle of West Nile virus.
Centre IRD Orléans, Technoparc—5 rue du Carbone, 45072 Orléans Cedex 2, France

Phone: +33 2 38 87 95 33 / 0 871 228 257; email: francois.baillon@ird.fr; Web site: http://balbuzard-migration.fr/

Anne-Laure Bañuls, PhD, works on the genetics and evolution of different pathogenic agents (*Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Toxoplasma gondii*), but especially of *Leishmania*.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: +33 4 67 41 62 99; email: Anne-Laure.Banuls@ird.fr; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Fernando Baquero, MD, PhD, born 1941, has worked on antibiotics-microbial organisms interactions for 40 years, with particular emphasis on genetics, ecology, and evolutionary biology of antibiotic resistance. He is Director of the Department of Microbiology at the Ramón y Cajal University Hospital in Madrid.
Department of Microbiology, Ramón y Cajal University Hospital, Carretera de Colmenar km 9,100, 28034 Madrid, Spain
Phone: +34 913368330; fax: +34 913368809; email: baquero@bitmailer.net

Philippe Barbazan, PhD, has worked for 8 years at the French Ministry of Foreign Affairs in West Africa and joined in 1988 IRD as a researcher in medical entomology. He as been appointed since 1998 at Mahidol University in Thailand as an expert for research in vector and vector control.
Institut de Recherche pour le Développement, IRD—Research Unit 178
Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, 25/25 Phutthamonthon 4, Nakhonpathom 73170, Thailand
Phone/fax: +66 2 441 0227; email: fnpbb@diamond.mahidol.ac.th; Web site: http://www.th.ird.fr

David G. Biron, PhD, works on the biochemical interactions in host–parasite systems especially with proteomics tools.
HPMTC Proteome Analysis, 223 St-Antoine, St-Elphège, QC, Canada, JOG1JO
Phone: +1(450)568–3568; fax: +1 (450)568–3568; email: d.biron@caramail.com

Jason K. Blackburn, PhD, began working with wildlife over a decade ago and has been working with GIS and disease applications for the past 6 years. He is an Assistant Professor in the Department of Geography at California State University, Fullerton, and his research focuses primarily on the use of ecological niche modeling methods and GIS-based analyses to study the spatial ecology of zoonotic diseases.
Department of Geography, California State University, Fullerton, 800 N. St. College Blvd., Fullerton, CA 92834-6846, USA
Phone: +1-714-278-3161; fax: +1-714-278-5223; email: jblack6@gmail.com; Web site: http://geography.fullerton.edu

Jean Blancou, DVM, PhD, has worked in African then in French national veterinary laboratories for 25 years. In 1990, he has been elected Director General of World Organisation for Animal Health (OIE), and has retired in 2000.
11, rue Descombes, 75017 Paris, France
Email: jblancou@noos.fr

Didier Bompangue, MD, born 1971, is epidemiologist. He has worked on response to epidemics in Democratic Republic of Congo with Médecins sans Frontières and with the health ministry. He is presently a PhD student at Franche-Comté University.
Service de Parasitologie, Hôpital Jean Minjoz, 25 000 Besançon, France
Phone: +33 3 81 66 80 68; email: joellebop@yahoo.fr

Rachel Bras-Gonçalves, PhD, born 1967, works in the "Pathogeny of *Trypanosomatidae*" laboratory. Her research focuses on the host immune responses.
IRD, Unit of Research "Facteurs moléculaires impliqués dans la maladie de Chagas et des leishmanioses)," BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 47; fax: +33 4 67 41 63 30; email: rachel.bras@ird.fr

Hélène Broutin, PhD, born 1977, focuses her research on the epidemiology and ecology of human infection diseases in the developing world. She is interested in the spatial and temporal dynamics of infectious diseases and their evolution under vaccine pressure. The main objective is to better understand emergence, persistence, and spread of diseases in order to adapt vaccine strategies for a better global prevention and control.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: + 33 4 67 41 62 99; email: broutinh@gmail.com; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Bruno Bucheton, PhD, born in 1971, is a human geneticist and has mainly worked on the genetic epidemiology of visceral leishmaniasis in East Africa (Sudan) and on the study of human macrophage responses to *Leishmania* infection in-vitro.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: + 33 4 67 41 62 99; email: bruno.bucheton@mpl.ird.fr; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Dominique Buchillet, PhD, is a medical anthropologist at the French Institut de recherche pour le développement. During the last 20 years, she has done extensive fieldwork on shamanism, epidemics, and indigenous health in the Brazilian Amazon. She is presently initiating research work on the history of epidemics and traditional medicines in Thailand and South China.
Institut de recherche pour le développement, Centre de Recherche IRD d'Île de France, 32, Avenue Henri Varagnat, 93143 Bondy Cedex, France
Phone: +33 1 48 02 79 20; fax: +33 1 48 02 79 44; email: Dominique.Buchillet@bondy.ird.fr; Web site: http:// www.ird.fr

Robin M. Bush, Ph.D., is an Associate Professor in the Department of Ecology and Evolutionary Biology at the University of California, Irvine.
59 Steinhaus, University of California, Irvine, CA 92697, USA
Phone: +1-949-824-2243; fax: +1-949-824-2181; email: rmbush@uci.edu; Web site: http://rmbush.bio.uci.edu/

Julien Cappelle, DMV, MSc, born 1981, is a PhD student on the environmental factors of AI persistence in African and Asian agro-ecosystems at ULB (Bruxelles) and CIRAD (Montpellier France). He is interested in the environmental factors of emergence and persistence of diseases.
CIRAD-Es TA C 22/E, Campus international de Baillarguet, 34398 St Gély-du-Fesc Cedex, France
Phone: +33 4 67593903; email: julien.cappelle@cirad.fr

Damien Chevallier, PhD student, born 1973, works on a project on the satellite tracking of Black storks *Ciconia nigra* and White storks *Ciconia ciconia* in Europe and West Africa in order to determine the consequences of the environmental changes on their breeding success.
Physiologie et Biologie des Organismes, Populations, Interactions, Institut de Recherche pour le Développement, IRD, CNRS—Institut Pluridisciplinaire Hubert Curien
Departement d'Ecologie, Physiologie et Ethologie, 23 Rue Becquerel, 67087 Strasbourg Cedex 2, France
Phone: +33 3 88 10 69 17; fax: +33 3 88 10 69 06; email: damienchevallier@yahoo.fr; Web site: http://www.latitude11

Marc Choisy, PhD, is interested in the understanding of the transmission dynamics of infectious diseases both in time and space. His work, essentially based on mathematical modeling and statistical analyses, has focused so far mainly on childhood diseases such as measles, whooping cough, or chickenpox in France and the United States.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: +33 4 67 41 62 99; email: marc.choisy@mpl.ird.fr; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Guillaume Constantin de Magny is an ex-PhD student of Jean-François Guégan, and a post-doc fellow (Assistant Researcher) at the University of Maryland Biotechnology Institute in Pr. Rita Colwell's research group.
University of Maryland Institute for Advanced Computer Studies, Center for Bioinformatics and Computational Biology, #296 Biomolecular Sciences Building—Room 3104H, College Park, MD 20742, USA
Phone: +1-301-405-3235; fax: +1-301-314-1341; email: g.demagny@gmail.com; Web site: http://www.cbcb.umd.edu/

Vaughn S. Cooper is an evolutionary biologist whose laboratory studies the genetics of adaptation to novel hosts by microbial populations, and in particular, the *Burkholderia cepacia* complex.
Department of Microbiology, University of New Hampshire, Durham, NH 03824, USA
Phone: +1-603-862-3422; fax: +1-603-862-2621; email: vaughn.cooper@unh.edu; Web site: http://microbiology.unh.edu/faculty/Cooper/

Jean-Paul Cornet, PhD, has worked on the arboviruses and other pathogenic tick-borne agents for 35 years. A former research engineer at the French Institut de Recherche pour le Développement (IRD), he is now retired.
"La Pastourelle," 75 parc gréco-romain, 83140 Le-Brusc, France
Phone: +33494340198; email: jean_paul.cornet@club-internet.fr

Andrew Curtis, PhD, born 1967, has worked on applying GIS and spatial analysis to public health concerns for 12 years. While director of the World Health Organization's Collaborating Center (WHOCC) for Remote Sensing and GIS for Public Health he coordinated projects in multiple countries. During this period, the WHOCC was also part of the Louisiana State University geospatial response to Hurricane Katrina. He now holds a position in the Department of Geography at the University of Southern California.
GIS Research Laboratory, Department of Geography, College of Letters, Arts and Sciences, University of Southern California, Kaprielian Hall (KAP), Room 416, 3620 South Vermont Avenue, Los Angeles, CA 90089-0255, USA
Phone: +1-213-740-8263; fax: +1 213-740-9687; email: acurti1@lsu.edu; Web site: www.usc.edu/dept/geography/gislab

Antoine Danchin, initially trained as a pure mathematician, is the Director of the Genomes and Genetics Department at the Institut Pasteur in Paris. His work focuses on gene organization in bacterial genome and their relationships with the architecture of the cell. After having been the creator of the HKU-Pasteur Research Centre in Hong Kong, he became interested in problems of epidemiology, in particular in the context of the emergence of diseases.

Genetics of Bacterial Genomes, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France
Phone: +33 1 45 68 84 42; fax: +33 1 45 68 89 48; email: antoine.danchin@normalesup.org; Web site: http://www.pasteur.fr/recherche/unites/REG/

Eric Delaporte, MD, PhD, born 1956, has worked on clinical and epidemiological characteristics of HIV infection in Africa for more than 20 years. He is professor of Tropical Diseases and head of a research unit of the French Institut de Recherche pour le Développement (IRD).
UMR 145: "HIV, AIDS and associated diseases", 911 avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 62 97; fax: +33 4 67 41 61 46; email: delaport@mpl.ird.fr; Web site: http://www.mpl.ird.fr

Jean-Pierre Dujardin, MD, D Sc, PhD, has worked on population biology and morphometrics of medically important insects for 25 years (http://www.mpl.ird.fr/mrphometrics).
Center of Excellence for Vectors and Vector Borne Diseases, Faculty of Sciences, Mahidol University, Nakhon Pathom, 73170 Thailand
Phone: +2885 1277; fax: +66 2 441 0227; email: jean-pierre.dujardin@ird.fr; Web site: http://www.th.ird.fr

Ananias A. Escalante, PhD, born 1962, has focused his research in the last 12 years on the evolutionary genetics of malarial parasites. He is associate professor at the School of Life Sciences, at Arizona State University, USA
School of Life Sciences, Arizona State University, PO Box 874501, Tempe AZ 85287-4501, USA
Phone: +1-480-965-3739; fax: +1-480-965-6899; email: Ananias.Escalante@asu.edu; Web site: http://sols.asu.edu/faculty/aescalante.php

Robert A. Feldman, PhD, born 1961, is the President and CEO of SymBio Corporation in Menlo Park, CA. His research and business interests include academic and biotech collaborations in ecological, evolutionary, and community genomics. Over the last 20 years, his work has included seminal studies in such diverse fields as conservation genetics of introduced pathogens of native Hawaiian forest birds, microbial symbionts of deep-sea hydrothermal vent invertebrates, and human microbiomics.
SymBio Corporation, 1455 Adams Dr., Menlo Park, CA 94025, USA
Phone: +1-650-321-7566; fax: +1-650-321-0129; email: rfeldman@sym-bio.com; Web site: http://www.sym-bio.com/

Ad Fluit, PhD, started at the Department of Physiological Chemistry at Utrecht University, and moved to the Eijkman-Winkler Institute where he started with DNA work; this is now extended to antibiotic resistance.
Eijkman-Winkler Institute, Rm G04.614, University Medical Center Utrecht., Heidelberglaan 100; 3584 CX Utrecht, P.O. Box 85.500; 3508 GA Utrecht, The Netherlands
Phone: +31 30 - 250.7625; fax: +31 30 - 254.1770; email: A.C.Fluit@umcutrecht.nl; Web site: http: //www.umcutrecht.nl

Florence Fournet is a researcher at the French Institute of Research for the Development, with a Ph.D. in Geography. She has developed original research projects on a comparative approach of urbanization and health disparities between West African and South East Asian cities.
IRD Burkina Faso, 01 BP 182, 01 Ouagadougou, Burkina Faso
Phone: +(226) 50 30 67 37: fax: (226) 50 31 03 85; email: fournet@ird.bf

Daniel N. Frank, PhD, is a research scientist in the Department of Molecular, Cellular, and Developmental Biology of the University of Colorado.
Department of Molecular, Cellular, and Developmental Biology, A115 Porter Biosciences, University of Colorado, Boulder, CO 80309-0347, USA
Phone: +1 303-735-1808; fax: +1 303-492-7744; email: Daniel.Frank@Colorado.EDU; Web site: http://mcdb.colorado.edu

Steven A. Frank, Ph.D., born 1957, works on the evolutionary genetics of infectious diseases and cancer. He is Professor of Biology at the University of California, Irvine.
Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697-2525, USA
Phone: +1-949-824-2244; fax: +1-949-824-2181; email: safrank@uci.edu; Web site: http://stevefrank.org

Christophe Fraser, PhD, born 1973, started in theoretical physics, and has worked on the theoretical biology of pathogens for 8 years. He is current Reader and Royal Society University Research Fellow in the Department of Infectious Disease Epidemiology at Imperial College London.
Department of Infectious Disease Epidemiology, Faculty of Medicine at St Mary's Campus, Imperial College London, London W2 1PG, UK
Phone: +44 20 7594 3397; fax: +44 20 7402 3927; email: c.fraser@imperial.ac.uk; Web site: http://www.imperial.ac.uk/medicine/people/c.fraser.html

Lionel Gagnevin, PhD, is a research scientist at Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) in Réunion Island, France. Research field: diversity and population genetics of *Xanthomonas*.
CIRAD, UMR Peuplements Végétaux et Bioagresseurs en Milieu Tropical CIRAD-Université de la Réunion, Pôle de Protection des Plantes, 7, chemin de l'Irat, 97410 Saint Pierre, Réunion Island, France
Phone: (+262) 262 49 92 00; fax: (+262) 262 49 92 93; email: lionel.gagnevin@cirad.fr; Web site: http://www.univ-reunion.fr/pvbmt/

Sylvain Godreuil, MD, PhD, works on nosocomial infections, antibiotic resistance, and on genetics and evolution of mycobacteria, especially *Mycobacterium tuberculosis*.
Laboratoire de Bactériologie, Hôpital Universitaire Arnaud de Villeneuve, 371 Avenue du Doyen Gaston Giraud, F-34295 Montpellier Cedex 5, France
Phone: 33 4 67 33 58 84; fax: 33 4 67 33 58 93; email: s-godreuil@chu-montpellier.fr

Jean-Paul Joseph Gonzalez, MD, PhD, leads a multidisciplinary research team on Disease Emergence. He dedicated his career, within the field of viral exotic diseases, for research, training, and expertise among many developing countries throughout the World.
Institut de Recherche pour le Développement, IRD—Research Unit 178, Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, 25/25 Phutthamonthon 4, Nakhonpathom 73170, Thailand
Phone/fax: +66 2 441 0227; email: frjpg@mahidol.ac.th; Web site: http://www.th.ird.fr

David E Gorla, PhD, born 1956, has worked on the ecology of Triatominae vectors of Chagas disease since 1978. Principal Researcher of the National Research Council of Argentina (CONICET) and director of CRILAR, a research scientific center of CONICET located in La Rioja (Argentina).
Inv Ppal CONICET, Entre Rios y Mendoza s/n, (5301) Anillaco—La Rioja (Argentina)
Phone: +54 (0)3827 494251; fax: +54 (0)3827 494231; email: dgorla@crilar-conicet.com.ar; Web site: http://www.crilar-conicet.gov.ar

Jean-François Guégan, PhD, is a Senior Research Director at the *Institut de Recherche pour le Développement*, the French Institute for Development. He is responsible for some courses (Masters and PhD degrees) in quantitative epidemiology and on global change and microorganisms, at Montpellier University.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: +33 4 67 41 62 99; email: jf.guegan@mpl.ird.fr; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Felipe Guhl, Full Professor Faculty of Sciences University of Los Andes, born 1949, has worked on epidemiology and molecular biology of tropical parasites transmitted by insect vectors with emphasis on Chagas Disease. Director of the Tropical Parasitology Research Centre, University of Los Andes. Member of the WHO Expert Advisory Panel on Parasitic Diseases.
Centro de Investigaciones en Microbiología y Parasitología, Tropical.Departamento de Ciencias Biológicas, Facultad de Ciencias, Universidad de los Andes, Bogotá, Colombia Carrera 1 No. 18A-10, Apartado Aéreo 4976, Bogotá, Colombia
Telefax: +57 1 3324540; email: fguhl@uniandes.edu.co; Web site: http://cimpat.uniandes.edu.co

William P. Hanage, PhD, works on the evolution and epidemiology of bacterial pathogens at Oxford University and, latterly, Imperial College London.
Imperial College Faculty of Medicine, Department of Infectious Disease Epidemiology, St Mary's Hospital, Norfolk Place, London W2 1PG, UK
Phone: +44 20 7594 3622; fax: +44 20 7594 3693; email: w.hanage@imperial.ac.u; Web site: http://www1.imperial.ac.uk/medicine/people/w.hanage.html

Heikki Henttonen is a Professor of Forest Zoology, in the Finnish Forest Research Institute (METLA). He is known all over the world as a specialist in Rodentology applied to Epidemiology.
Finnish Forest Research Institute, Vantaa Research Centre, POB 18, FIN-01301 Vantaa, Finland

Vincent Herbreteau PhD student, born 1975, works on the conditions and dynamics of the emergence of rodent-borne diseases in Thailand.
Laboratoire Espace, Santé, Territoire; Université Paris X-Nanterre, 200 avenue de la république, 92000 Nanterre, France
Email: Vincent.Herbreteau@ird.fr

Jean-Louis Herrmann, PharmD, PhD, born 1959, has worked on host/microbial pathogens interactions, and now for the last 10 years on mycobacteria (*M. tuberculosis*, *M. abscessus*). He is presently Professor of Microbiology at the Paris Ile de France Ouest University in Raymond Poincaré hospital.
Service de Microbiologie, Hopital Raymond Poincaré, 104 Bd Raymond Poincaré, 92380 Garches, France
Phone: +33 1 47 10 44 70 or 79 50; fax: +33 1 47 10 79 49; email: jean-louis.herrmann@rpc.aphp.fr; Web site: http://www.sls.ap-hop-paris.fr/

Mallorie Hide, PhD, studies the growth dynamics, molecular evolution, and proteomics of *Leishmania* parasites.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: +33 4 67 41 62 99; email: mallo.hide@mpl.ird.fr; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Austin L. Hughes, PhD, is Professor of Biological Sciences and Director of the Institute of Biological Research at Technology at the University of South Carolina. He is a pioneer and leader in bioinformatics on the adaptive evolution at the molecular level.
Department of Biological Sciences, University of South Carolina, Columbia SC 29208 USA

Phone: +1-803-777-9186; fax: +1-803-777-4002; email: austin@biol.sc.edu; Web site: http://www.biol.sc.edu/faculty/hughes.html

Jean-Pierre Hugot, DVM, PhD, is a parasitologist and a specialist in coevolutionary studies. He has spent most of his career in the French Museum of National History. Since several years he works in Asia where he runs a Franco-Thai program (*Rodents and Hantavirus in South Asia*) and coordinates the Binational Thai-French Joint Master: "*Infectious Diseases and Food Safety Evolution, Emergence, Spread and Control*."
Museum National d'Histoire Naturelle, Origine, Structure et Evolution de la Biodiversité, UMR 5202 du CNRS / USM 601, 55, rue Buffon, 75231 Paris Cedex 05, France
Phone: +33 1 40 79 35 05; email: hugot@cimrs1.mnhn.fr; Web site: http://www.mnhn.fr/mnhn/meo/

Shaden Kamhawi, PhD, born 1963, has worked on epidemiology and vector biology of leishmaniasis for over 17 years. Presently a core staff scientist at the NIH.
Laboratory of Malaria and Vector Research, NIAID/National Institutes of Health, Twinbrook III, 12735 Twinbrook Parkway, Room 2E-32, Bethesda, MD 20892-8132, USA
Phone +1-301-451-7922; fax: +1-301-594-5373; email: SKAMHAWI@niaid.nih.gov

Altaf A. Lal, PhD, MSc, born 1954, has worked on molecular biology and evolutionary genetics of parasitic agents and development and evaluation of vaccines against malaria for the last 25 years. He is presently serving as the Health Attaché, Embassy of the United States of America and the U.S. Department of Health and Human Services representative for South Asia. He served as the Chief of Molecular Vaccine Section, Division of Parasitic Diseases, Centers for Disease Control and Prevention.
Embassy of the United States of America, Shantipath, Chanakyapuri, New Delhi 110021, India
Phone: +91-11-2419-8213; fax: +91-11-2419-8770; email: Lala@state.gov

Philippe Henri Lagrange, MD, born 1939, has work on microbiology and immunology of mycobacterial diseases (tuberculosis and leprosy) for more than 30 years. He is presently working as consultant in microbiology at the Saint Louis Hospital of Paris.
Service de Microbiologie, Hôpital Saint Louis, 1 Avenue Claude Vellefaux, 75475 Paris Cedex 10, France
Phone: +33 1 42 38 53 79; fax: +33 1 42 49 92 00; email: philippe-henri.lagrange@sls.ap-hop-paris.fr

Christian Laurent, PhD, works on epidemiology and clinical research on human immunodeficiency virus infection in Africa.
UMR 145: "HIV, AIDS and associated diseases," 911 avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 (0)4 67 41 62 97; fax: +33 (0)4 67 41 61 46; email: Christian.Laurent@mpl.ird.fr; Web site: http://www.mpl.ird.fr

Pierre-Charles Lefèvre, DVM, PhD, has worked in Central America (Guatemala) and Africa (Ethiopia, Chad, Senegal). Back to France, he has worked for a research institute on animal tropical diseases before joining the French Ministry of Agriculture and in 1998 Fisheries.
127, rue d'Avron, 75020 Paris, France
Email: pocolo@wanadoo.fr

Meriadeg Le Gouilh, PhD student, mammalogist, works on ecological and evolutionary mechanisms surrounding the implication of bats in emergence and functioning of viruses in Thailand.
Unité 178 Conditions et Territoires d'Emergence des Maladies, Institut de Recherche pour le Developpement, IRD, Faculty of Sciences, Sciences bldg. 1-2, MU, Salaya 999 Phutthamonthon 4, Nakhonpathom 73170, Thailand
Phone fax: +(66) 2 441 02 27; email: fnmlg@diamond.mahidol.ac.th

Jean-Loup Lemesre, PhD, born 1955, has worked on biology and immunology of parasitic diseases for 25 years. A director of research at the French Institut de Recherche pour le Développement (IRD) he is presently responsible of a research team in charge of a research sector on Leishmaniasis.
IRD, Unit of Research "Facteurs moléculaires impliqués dans la maladie de Chagas et des leishmanioses)", BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 47; fax: +33 4 67 41 63 30; email: lemesre@mpl.ird.fr

Eric Leroy, DVM, PhD, born 1965, is presently the Head of Unit of Emerging Diseases (Chef du Service des Maladies Emergentes), Centre International de Recherches Médicales de Franceville, BP 769 Franceville, Gabon.
CIRMF, BP 769, Franceville, Gabon
Phone: +(241) 05 81 02; fax: +(241) 67 72 95; email: Eric.Leroy@ird.fr

Hugh D. Loxdale, PhD, is mainly working on the population genetics of aphids and other small insects using molecular markers.
Friedrich Schiller University, Institute of Ecology, Dornburger Str. 159, 07743 Jena, Germany
Phone: +49 3641 949 49448; fax: +49 3641 949 402; email: Hugh.Loxdale@uni-jensa.de; Web site: http://www2.uni-jena.de/biologie/ecology/multi_members/Loxdale/

Kate McGee is a doctoral student in the Bioinformatics Research Program at North Carolina State University, enrolled in 2004. Her doctoral work involves developing and using methods to understand the evolutionary dynamics of recombination and demography in model systems including humans and malaria.

Bioinformatics Research Center, North Carolina State University, Raleigh, NC 27695-7614, USA
Email: kmmcgee2@ncsu.edu

Natalia Mantilla-Beniers, born 1974, is interested in the ecology, spread and evolution of all things transmissible (e.g., infections, ideas). She has explored the population ecology of sympatric childhood diseases by contrasting historical disease records to predictions from various types of mathematical mechanistic models. Currently, she studies infections that can be acquired repeatedly and the problems associated to their control.
Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portugal
Phone: +351 214 407 923; fax: +351 21 4407970; email: nmantilla@igc.gulbenkian.pt; Web site: http://www.igc.gulbenkian.pt

Alvaro Moncayo, MD, PhD, has worked from 1979 to 2001 as Chief of the Programme of Research and Control of Chagas Disease at the World Health Organization (TDR) in Geneva, Switzerland. Currently he is an Associate Researcher at the CIMPAT.
CIMPAT, Universidad de los Andes, Calle 70 No. 5-60, Bogotá, DC, Colombia
Phone: +57.1.210.1881; fax: +57.1.332.4540; email: amoncayo@uniandes.edu.co

Bernard Mondet, PhD, born 1948, is a medical entomologist at the Institut de Recherche pour le Développement (IRD). Research studies in South America and West Africa on biology and ecology of blackflies, tse-tse flies and, for 20 years, on biology and eco-physiology of mosquitoes vectors of arbovirus. Presently in charge of a research program on urbanization and vector-borne diseases in South India at the French Institute of Pondicherry (IFP), India.
Institut Français de Pondichérry, CP 33, 11, Saint-Louis Street, Pondicherry, 605-001, India
Phone: +91 0413 – 233 41 68; email: bernard.mondet @ifpindia.org; Web site: www.ifpindia.org

Janice Moore, PhD, has worked on parasite-induced host behaviors for 30 years. She is Professor of Biology at Colorado State University.
Biology Department, Colorado State University, Fort Collins CO 80026, USA
Phone: +1-970-491-7011; fax: +1-970-491-0649; email: janicemoore@comcast.net; Web site: http://rydberg.biology.colostate.edu/faculty/moore.html

Stephen A. Morse, MSPH, PhD, born 1942, has worked in the areas of microbial physiology and pathogenesis for 38 years. He is presently the Associate Director for Science, Division of Bioterrorism Preparedness and Response and the Director of the Environmental Microbiology Program at the Centers for Disease Control and Prevention.
Associate Director for Science Bioterrorism Preparedness and Response Program, Centers for Disease Control and Prevention, 1600 Clifton Rd, MS C-18, Atlanta, Georgia 30333, USA
Phone: +1-404 639-3559; fax: +1-404-639-0382; email: sam1@cdc.gov; Web site: http://www.cdc.gov/

Hercules Moura, PhD, is involved in projects to develop and improve methods for detection and identification of adducts of chemical warfare agents NCEH, CDC, Sciences, Atlanta, GA, USA, Emergency Response and Air Toxicants Branch, Division of Laboratory Sciences, M.S. F-44, Chamblee Campus, Bldg. 110, Room 5112E, National Center for Environmental Health.
Centers for Disease Control and Prevention, 4770 Buford Hwy NE, Atlanta, GA 30341-3724, USA
Phone: +1-770-488-7843; fax: +1 770-488-0509; email: Hmoura@cdc.gov; Web site: http://www.cdc.gov/

Narong Nitatpattana, PhD, born 1964, has worked for about 20 years, mostly on Dengue and Japanese Encephalitis vaccine development at the Center for Vaccine Development, Institute of Science and Technology for Research and Development, Mahidol University, Thailand. He has developed for height years a Center on Emerging Viral Diseases in partnership with the Institute de Recherche pour le Développement (IRD). He is presently the head of the Inactivated Tissue Culture Derived of Japanese Encephalitis Vaccine project.
Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, 25/25 Phutthamonthon 4, Nakhonpathom 73170, Thailand
Phone/fax: +66 2 441 0227; email: frjpg@mahidol.ac.th; Web site: http://www.th.ird.fr

Martine Peeters, PhD, born 1958, has worked on genetic diversity and evolution of HIV for more than 20 years. Director of research at the French Institut de Recherche pour le Développement (IRD).
UMR 145: "HIV, AIDS and associated diseases," 911 Avenue Agropolis, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 (0)4 67 41 62 97; fax: +33 (0)4 67 41 61 46; email: Marine.Peeters@mpl.ird.fr; Web site: http://www.mpl.ird.fr

Renaud Piarroux, MD, PhD, born 1960, is both pediatrician and microbiologist. He has worked on ecology of infectious disease for 15 years and, with the NGO Médecins du Monde, took part in humanitarian emergencies in numerous countries. He is presently heading a research team called "Health and Rural Environment" at Franche-Comté University.

Service de Parasitologie, Hôpital Jean Minjoz, 25 000 Besançon, France
Phone: 33 3 81 66 80 68; email: renaud.piarroux@univ-fcomte.fr

Robert Poulin, PhD, born 1963, has worked on the evolutionary ecology of parasitism for 20 years. He is Professor of Zoology at the University of Otago, in New Zealand.
Department of Zoology, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand
Phone: +64 3 479-7983; fax: +64 3 479-7584; email: robert.poulin@stonebow.otago.ac.nz; Web site: http://www.otago.ac.nz/parasitegroup/home.html

Olivier Pruvost, PhD, is a research scientist at CIRAD in Réunion Island, France. His research field is epidemiology of diseases caused by *Xanthomonas* spp.
CIRAD, UMR Peuplements Végétaux et Bioagresseurs en Milieu Tropical CIRAD-Université de la Réunion, Pôle de Protection des Plantes, 7, chemin de l'Irat, 97410 Saint Pierre, Réunion Island, France
Phone: +262 262 49 92 00; fax: +262 262 49 92 93; email: olivier.pruvost@cirad.fr; Web site: http://www.univ-reunion.fr/pvbmt/

Stephane Rican, PhD, born 1971, work on health spatial inequalities in France. Assistant Professor in health geography at University Paris X-Nanterre.
Université Paris X-Nanterre, UFR SSA, Laboratoire Espace, Santé et Territoire, 200 Avenue de la République, 92000 Nanterre, France
Phone: +33 1 41 10 14 75; fax: +(33) 1 41 10 14 59; email: srican@u-paris10.fr

Pejman Rohani, born 1970, focuses his research on the population dynamics of host-natural enemy interactions, with a special focus on the ecology of human infection diseases. He is interested in the dynamical effects of stochasticity, seasonality, and nonlinearity and their public health implications.
Institute of Ecology, University of Georgia, Athens, GA 30602, USA
Phone: +1-706-542-9249; fax: +1-706-583-0132; email: rohani@uga.edu; Web site: http://www.uga.edu/rohanilab

Philippe Roumagnac, PhD, works on evolution and population genetics of *Salmonella Typhi* and *Yersinia pestis*.
Max-Planck Institut für Infektionsbiologie Schumannstr, 21/22 10117 Berlin, Germany
Phone: +49 30 28460202; fax: +49 30 28460111; email: roumagnac@mpiib-berlin.mpg.de; Web site: http://web.mpiib-berlin.mpg.de/ mlst/AGroup

Gérard Salem, PhD, MSc, born 1952, has worked on relation between Urbanization and Health in Africa, Asia, and France as a medical geographer. He is presently Professor at the University Paris X-Nanterre and Head of the French Observatory of Cancers (Institut National du Cancer).
Université Paris X-Nanterre, UFR SSA, Laboratoire Espace, Santé et Territoire, 200 Avenue de la République, 92000 Nanterre, France
Phone: +33 1 40 97 75 53; email: gsalem@ext.jussieu.fr; Web site: http://www.u-paris10.fr

Yerlan Sansyzbayev, PhD, has worked on disease immunology, epidemiology, and GIS for several years at the Kazakh Science Center for Quarantine and Zoonotic Diseases. Today he is the Biological Threat Reduction Program Medical Coordinator for the Technology Management Company in Almaty, Kazakhstan.
Technology Management Company, 29/6 Akademik Satpaev St., 5th floor, Business Tower, Hyatt Hotel, Almaty, Kazakhstan 050040
Phone: +7 3272 980 228/229; fax: +7 3272 980 230; email: ysansyzb@bechtel.com

Serge Savary, PhD, is a plant pathologist at the Institut National de la Recherche Agronomique, France. Much of his research has been conducted in the developing world and has dealt with the quantification and modeling of botanical epidemics and crop losses in agroecosystems. He currently is head of the INRA Plant Health Unit in Bordeaux, France.
INRA, 71 ave. E. Bourleaux, UMR Santé Végétale, B.P. 81, 33883 Villenave d'Ornon Cedex, France
Email: ssavary@bordeaux.inra.fr

Chris J. Schofield, DSc, PhD, FRES, Coordinator of the ECLAT Network, has worked on the biology and control of Triatominae and Chagas disease for over 30 years. He also coordinates the LTTRN network for research and control of tsetse and trypanosomiasis in Africa.
London School of Tropical Medicine, Keppel Street, WC1E7HT London, UK
Phone: +44 207 927 2340; fax: +33 4 50.20.63.77; email: cj.schofield@wanadoo.fr; Web site: http://ECLAT.fcien.edu.uy/

Dennis E. Slice, born 1958, specializes in the development and application of tools for morphometric analysis. His work ranges from software development to research in forensic anthropology, ecology, and evolutionary biology. For a number of years, he has taught morphometrics courses at the University of Vienna.
Department for Anthropology, University of Vienna, Althanstrasse 14, A-1091 Vienna (Austria)
Fax: +43-1-4277-9547; email: dslice@morphometrics.org; Web site: http://dslice.web.aplus.net

Marc Souris, PhD, born 1954, develops algorithms, methodology, and software in the field of geographic information systems and remote sensing since 1983. He is the principal author of the SavGIS global GIS software solution. He works for urban planning, risk analysis, and mitigation for natural disasters, and spatial and environmental epidemiology and disease emergence modeling.

Center for Vectors and Vector-Borne Diseases, Faculty of Science, Mahidol University, 25/25 Phutthamonthon 4, Nakhonpathom 73170, Thailand
Phone/fax: (662) 441 0227; email: souris@ird.fr; Web site: http://www.th.ird.fr

Brian Geoffrey Spratt, PhD, Professor of Molecular Microbiology and Wellcome Trust Principal Research Fellow, Department of Infectious Disease Epidemiology, Imperial College London, London W2 1PG, UK. Brian Spratt has worked since the late 1980s on molecular epidemiology and the population and evolutionary biology of bacterial pathogens. He is Head of the Department of Infectious Disease Epidemiology at Imperial College London.
Department of Infectious Disease Epidemiology, Imperial College London, St Mary's Hospital Campus, London W2 1PG, UK
Phone: +44 20 7594 3398; fax: + 44 20 7402 3927; email: b.spratt@imperial.ac.uk; Web site: http://www1.imperial.ac.uk/medicine/people/b.spratt/

Jamie R. Stevens, PhD, born 1962, has worked on evolutionary genetics of parasites and pathogens for over 20 years. Is currently an Associate Professor in the School of Biosciences at the University of Exeter, UK where he researches and lectures in evolution, parasitology and bioinformatics.
Hatherly Laboratories, School of Biosciences, University of Exeter, Prince of Wales Road, Exeter EX4 4PS, UK
Phone: +44 1392 263775; fax: +44 1392 263700; email: j.r.stevens@ex.ac.uk; Web site: http://www.people.ex.ac.uk/jrsteve0

Yupin Suputtamongkol is a Doctor in Tropical Medicine. She is a staff member in the Division of Infectious Diseases and Tropical Medicine, at the Faculty of Medicine of Siriraj Hospital (Mahidol University, BKK). Dr Suputtamongkol is known worldwide as a specialist in Leptospirosis and Murine Typhus.
Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Xin-zhuan Su, PhD, uses the parasite genome databases to develop genome-wide polymorphism maps to study the population structure, evolution history, transmission dynamics, and mechanisms of drug resistance. He is currently the head of the Malaria Functional Genomics Section and a Senior Investigator.
National Institute of Allergies and Infectious Disease, National Institute of Health, 12735 Twinbrook Parkway, Bethesda, MD 20850, USA
Phone: +1-301-402-0876; fax: +1-301-402-2201; email: xsu@mail.nih.gov; Web site: http://www3.niaid.nih.gov/labs/aboutlabs/lmvr/malariaFunctionalGenomicsSection/

Shyam Sundar, MD, born 1953, has worked on leishmaniasis for 20 years. He is a Professor of Medicine.
Institute of Medicial Sciences, Banaras Hindu University, Varanasi 221 005, India
Phone: +91-542-2309493; fax: + 91-542-2367568; email: drshyamsundar@hotmail.com; Web site: http:// www.bhu.ac.in

Loubna Tazi, PhD, works currently as a postdoctoral researcher at Brigham Young University in the group of Dr. Keith A. Crandall. Her projects focus on population and evolutionary genetics, and molecular epidemiology of microbial populations.
Brigham Young University, Department of Microbiology and Molecular Biology, 751 Widtsoe Building, Provo, UT 84602, USA
Phone: +1-801-422-2506; fax: +1-801-422-0090; email: lt247@byu.edu

Frederic Thomas, PhD, born 1970, has worked on the evolutionary ecology of parasitism since 10 years. He is director of Research at CNRS.
Genetics and Evolution of Infectious Diseases, UMR CNRS/IRD 2724, BP 64501, 34394 Montpellier Cedex 5, France
Phone: +33 4 67 41 61 97; fax: + 33 4 67 41 62 99; email: frederic.thomas@mpl.ird.fr; Web site: http://gemi.mpl.ird.fr/cepm/index.htm

Myrtille Tibayrenc, born 1980, graduated from the Ecole des Beaux-Arts in France. She is presently an artist and a cultural officer at the Alliance française in Bangkok, Thailand (art management).
Alliance française, 29 Thanon Sathorn Tai, Bangkok 10120, Thailand
Phone: +66 2 670 4224; fax: +66 2 679 2055; email: mimi@alliance-francaise.or.th; Web site: http://www.alliance-francaise.or.th

Charles W. Todd, PhD, born 1953, has worked on tropical disease research and vaccine development for almost 30 years.
Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA, 30341, USA
Phone: +1-770-488-4526; fax: +1-770-488-4253; email: ckt1@cdc.gov

Walairut Tuntaprasart, PhD, born 1972, works on mosquito-borne diseases and vector control, including molecular genetics, environment and public health aspects. Currently, she works at the Faculty of Public Health, Mahidol University and has a jointed project with the Institute de Recherche pour le Developpement (IRD).
Faculty of Public Health, Mahidol University, 420/1 Rajvithee Rd., Ratchathewee, Bangkok 10400 Thailand
Phone: +66 2 644 6842-5 ext. 1202; fax: +66 2 644 5130; email: phwtp@mahidol.ac.th; Web site: http://www.mahidol.ac.th/

Gabriel Turinici is currently Professor of Mathematics at the Paris Dauphine University. His research focuses on numerical simulations and scientific computing. Domains of applications range from laser-matter interaction (also known as "quantum control") and social sciences to epidemic propagation.
CEREMADE, University of Paris IX Dauphine, Place du Marechal De Lattre De Tassigny, 75775 Paris Cedex 16, France
Phone: +33 1 44 05 48 58; fax: +33 1 44 05 45 99; email: Gabriel.Turinici@dauphine.fr; Web site: http://www.ceremade.dauphine.fr/~turinici/

Venkatachalam Udhayakumar, PhD, is the Chief of the Genetics and Immunology Laboratory.
Malaria Branch, Division of Parasitic Diseases, National Center for Zoonotic, Vector-Borne and Enteric Diseases, Centers for Disease Control and Prevention, U.S. Public Health Service, Atlanta, GA 30347, USA
Phone: +1-770-488-4047; fax: +1-770-488-4454; email: vxu0@cdc.gov

Jan Verhoef, MD, PhD, became Professor of Clinical Microbiology and Infectious Diseases in 1977 and chairman of the Eijkman-Winkler Institute in 1978. He was the first president of the ESCMID (European Society of Clinical Microbiology and Infectious Diseases).
Eijkman-Winkler Institute, Rm G04.614, University Medical Center Utrecht., Heidelberglaan 100; 3584 CX Utrecht, P.O. Box 85.500; 3508 GA Utrecht. The Netherlands
Phone: +31 30 - 250.7625; fax: +31 30 - 254.1770; email: J.Verhoef@umcutrecht.nl;Web site: http: // www.umcutrecht.nl

Kathleen Victoir, PhD, born 1969, has worked on infectious diseases for more than 10 years as a scientist, a consultant, and a scientific officer. Presently in charge of a program on infectious diseases and innovation for a European network.
Pasteur Institute, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France
Phone: (+33) 1 44 38 91 15; email: kvictoir@pasteur.fr

Pierre Vidal is a French anthropologist. He has dedicated his career to the ethnography and archeology of the populations of Central Africa. He retired as researcher from the French Foreign Office and he is presently a permanent secretary of the Pays de Sault Historical Society, France.
Le Village, 11340, Roquefeuil, France
Phone: +33 468693742; email: pierre-vidal2@wanadoo.fr

Yannick Jaffré, PhD, has worked as anthropologist in West Africa for 20 years. He collaborated with public health teams, conducted many anthropological researches focused on health priority, and teached in many African and French medical faculties. He is now responsible of the PhD teaching program in health anthropology in SHADYC (Sociology, History and Anthropology of Cultural Dynamics) in a French social sciences high school (EHESS). He wrote many books and articles about West African conceptions of diseases, and the relationships between healthcare providers and users.
SHADYC (EHESS-CNRS), Centre de la Vieille Charité, 13002 Marseille (France)
Phone: +33 4 91 14 07 54; email: jaffre@univmed.fr

Mario Ortiz Yanne, PhD student, has conducted applied research on the epidemiology, entomology and control of Dengue Fever in an endemic area in Colombia.
Department of Biology, School of Sciences, Universidad de los Andes, Calle 70 No. 5-60, Bogotá, DC, Colombia
Phone: +57.1.210.1881; fax: +57.1.332.4540; email: mario-or@uniandes.edu.co

Kumiko Yoshimatsu is working in the Institute for Animal Experimentation, Hokkaido University, School of Medicine, Sapporo, Japan. She is known worldwide for her works on hantaviruses in Japan and in South Asia.
Institute for Animal Experimentation, Hokkaido University, School of Medicine, Sapporo, Japan

Biographical

Michel Tibayrenc was born in 1947 in a dirty suburb of Paris, which adorns his spoken French with the equivalent of the cockney London accent. Possibly to escape that grey and sad environment, he was soon attracted to a professional carrier in sunny countries. He became a medical doctor at the university of Paris in 1975 and started his career as a general practitioner in Algiers and Paris. With a frustrated vocation as a scientist and even further as a naturalist, he turned to scientific disciplines with medical relevance (parasitology and medical entomology), and received his Ph.D. from the university of Paris in 1986. A full-time researcher at the Institut de Recherche pour le Développement (see introductory chapter) since 1977, he has participated in field-work in both French Guiana (one year) and Bolivia (5 years). Then in 1985, a large cultural leap sent him to the university of California at Davis, at the laboratory of the renowned evolutionist Francisco J. Ayala (Chapter 11), with whom he started a fruitful collaboration. Other stays in America include UC Irvine (Francisco Ayala's new location) and the Centers for Disease Control (CDC) in Atlanta. Head of the laboratory of Genetics and Evolution of Infectious Diseases at the IRD center of Montpellier (France) from 1986 to 2005, he is now the IRD representative in Bangkok.

Michel Tibayrenc is the initiator, with CDC's Altaf Lal (see Chapter 8), of the MEEGID congresses (Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases). He is the founder and editor-in-chief of the journal *Infection, Genetics and Evolution* (Elsevier; http://www.elsevier.com/ locate/meegid) and in 1997 introduced the concept of a European CDC. In 2005, he launched an international think tank, Biology, Medicine, and Society, to think about the social implications of modern biological and medical research.

Divorced, he has been highly successful in persuading his three children to embrace any job but scientific research.

Hobbies include contemporary history, natural history, karate (a former professional trainer), old motorcycles, and dancing (he is now professor of the dancing club of the Alliance Française in Bangkok).

Address:
IRD Representative Office
French Embassy
29, Thanon Sathorn Tai,
Bangkok 10120, Thailand
Tel: + (66 2) 627 2190
Fax: + (66 2) 627 2194
Cel: + (668 1) 82 64 056
E-mail:
(direct): Michel.Tibayrenc@ird.fr
(secretary) ird_th@ksc.th.com
Web site: http://www.th.ird.fr

Introduction: Infectious Diseases, the Major Challenge of Twenty-First Century Medicine

How Should Humans, Domestic Animals, and Crops Be Protected?

THE DARK AGE OF INFECTIOUS DISEASES

The title of this introductory chapter would have amazed the medical student I was in the 1970s. In that pre-AIDS era of medicine, infectiology was the emblem of triumphant medicine, with the icon names of Jenner, Pasteur, Koch, and Flemming, among others. The student who chose to specialize in this field appeared to be opting for the easy road to fame and fortune. This is no longer true and in the past has been so only for the industrial world. The peril of emerging and reemerging infectious diseases (ERID) was clearly identified by the US health and political authorities more than 10 years ago [1]. This was before the mad cow disease, foot-and-mouth disease, SARS, and avian flu affairs. At present, we have entered the dark age of infectious diseases [9]. Africa is ravaged with what have been called the three diseases of poverty—malaria, tuberculosis, and AIDS—as well as many others. Southeast Asia is threatened by major pandemics (SARS, avian flu). Latin America continues to be heavily struck by Chagas disease, a parasitic disease transmitted by true bugs that proliferate in poor habitats. The industrial world is not spared. In New York City, 25% of the strains of *Mycobacterium tuberculosis*, the bacillus responsible for tuberculosis, are resistant to most antibiotics. In France, several thousand people die every year from hospital infections. Finally, the European Union has now been reached by avian flu.

Many experts agree that the threat of a major pandemic, comparable to the Spanish flu at the end of the First World War (which killed millions of people) is growing [3,5].

It is therefore clear that the infectious threat today is no longer under control. The publication of this book is hence quite timely in exposing the different facets of modern research in this key domain of infectiology and the measures that can be envisaged to better fight against this peril.

Fig. 1. Views of fieldwork in Bolivia. Left: The author's car on a precarious bridge; middle: the road between La Paz and the tropical valleys has just collapsed (*derrumbe*). Truck drivers and the author have to wait hours for the road services bulldozer to repair the road. Right: The author in the tropical valleys near La Paz helped in his field studies on Chagas disease by very helpful and friendly young Aymara Indians.

NEED FOR A PRAGMATIC, MULTIDISCIPLINARY APPROACH

My background is hard science, by opposition to human sciences (anthropology, ethnology, sociology). I am more precisely a bench scientist (we give ourselves the nickname of lab rats). However, I do have experience as a clinician, and long experience as a field researcher in southern countries (Figure 1).

My knowledge of the southern parts of the planet through long stays (nearly 10 years) include Algeria, French Guiana, Bolivia, and now Thailand. My experience also includes 4 years in the United States, in the most modern laboratories of that country, the Universities of California, and the Centers for Disease Control (CDC) in Atlanta (see biographical sketch). This contrasted, dual experience has put me in contact with the reality of infectious diseases and epidemics and has brought me face to face with the abyss that stands between basic research and the pressing needs of populations suffering from high-load infectious problems. I am not advocating that upstream research should be abandoned. On the contrary, I am more than ever convinced that a major effort in this direction should be made, for it is often the shortest way toward practical medical applications. But this confrontation has convinced me that a pragmatic view to the ERID problem is necessary, which involves a multidisciplinary and holistic approach. All disciplines should contribute to this fight, with no ostracism and no hierarchization. Of course **high-tech research** is prestigious and fascinates young scientists, and for good reason. It is also our best hope for radical solutions to the infectious peril. However, so-called **old-fashioned savoir-faires, such as traditional medical entomology and microbiology**, remain indispensable and are only too often

IRD, Institut de Rercherche pour le Développement, formerly ORSTOM, Office de Recherche Scientifique et Technique Outre-Mer, a French governmental organization for research in developing countries (http://www.ird.fr) ; the author has been working here since 1977.

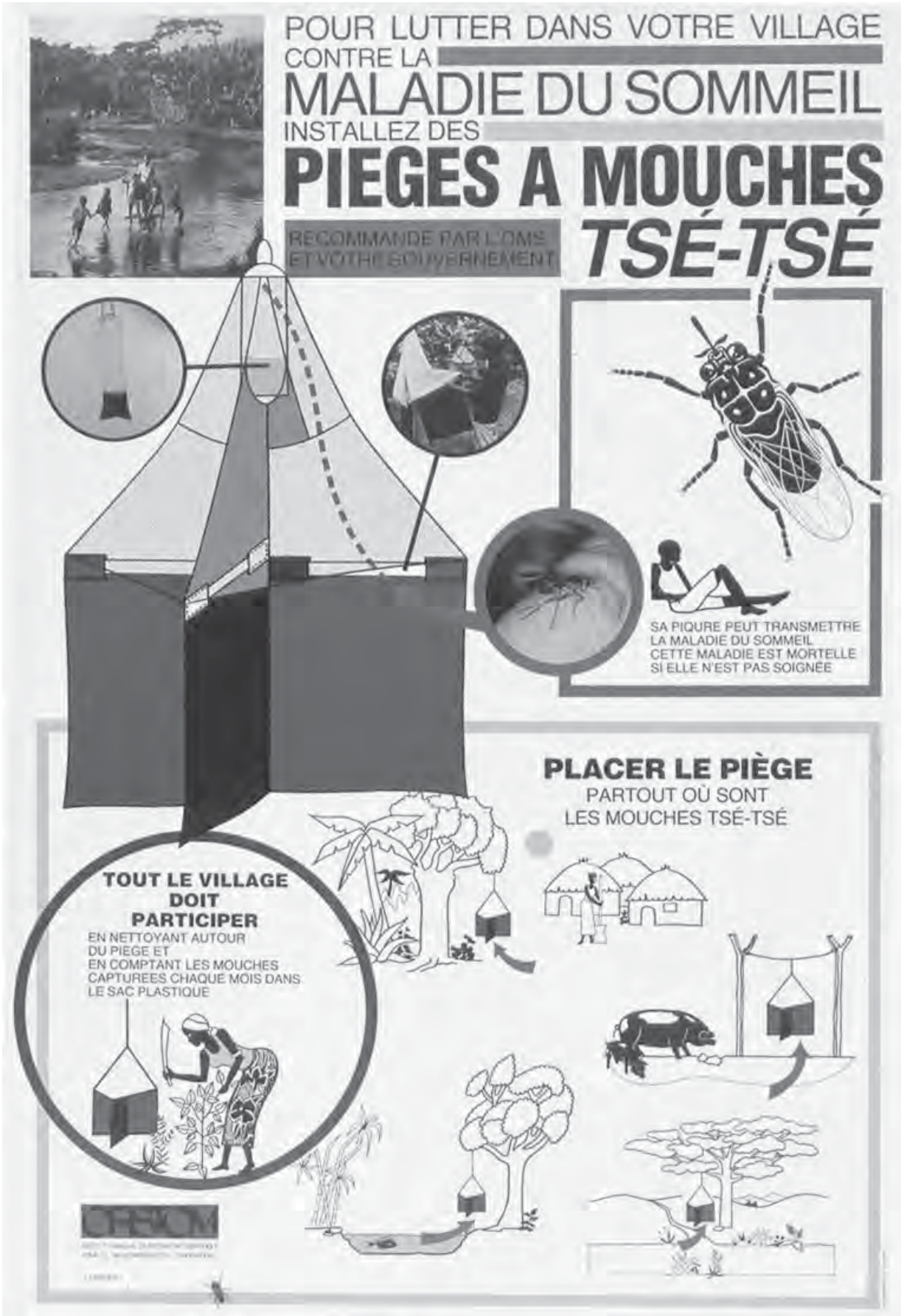


Fig. 2. An informational poster circulated among rural populations in French-speaking African countries to encourage people to use antitsetse fly traps (credit IRD).

despised by young talents [11]. Chapter 25 shows that they can be updated by modern technologies. Moreover, a **thorough knowledge of the field** is crucial for understanding infectious disease epidemiology and for controlling these diseases. There is no lack of examples where very simple measures inspired by this deep knowledge of the field have been more efficient in controlling diseases than high-tech tools. Tsetse flies (see Figure 7, Chapter 19) are the vectors of sleeping sickness (now called human African trypanosomiasis) in Africa. The very simple Challier-Laveyssiére tsetse fly trap (Figure 2), invented by two IRD colleagues of mine, is a typical case of a simple and efficient technology that has emerged from field work. It relies on a very simple principle, which was empirically discovered: tsetse flies are strongly attracted to the color blue, as is the bull to the color red. The trap is simply composed of a piece of blue fabric under a cone of gauze. The flies are attracted by the blue fabric and then climb toward the light, at the top of the trap, where a small insecticide reservoir is waiting for them. The trap was first designed to catch the flies for surveys and studies. But it appeared that the number of flies caught was so high that only one trap per village was enough to significantly lower the level of transmission of HAT, provided that well-adapted information was circulated among populations (Figure 2).

ENVIRONMENTAL VERSUS BIOLOGICAL PARAMETERS

The holistic approach exposed above strongly implies that in-built biological parameters are not the only ones to be considered.

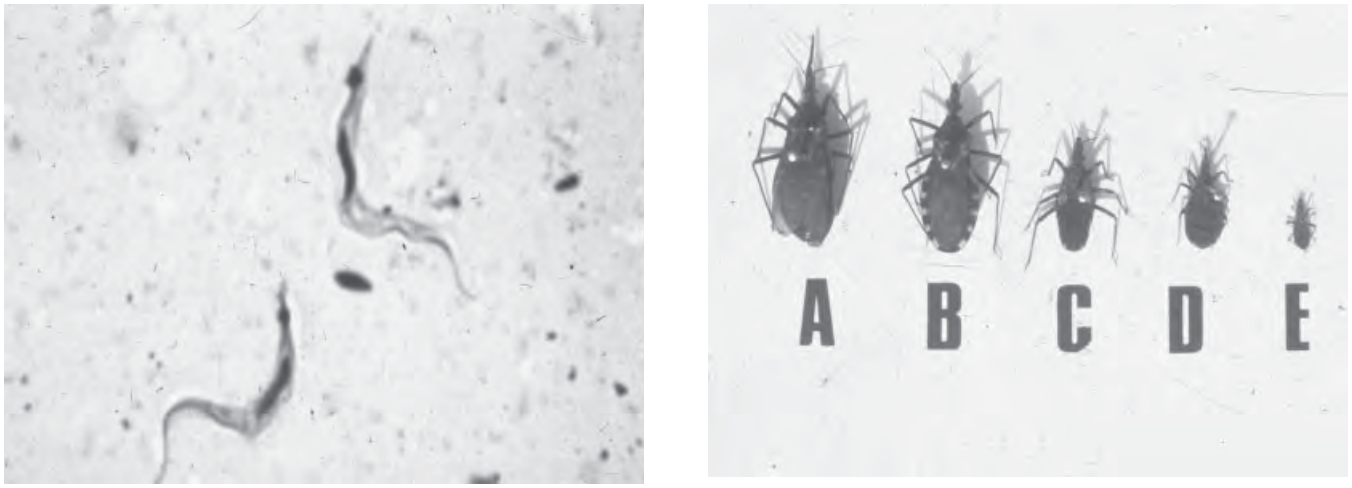
Tuberculosis, AIDS, and malaria have been identified as the three diseases of poverty by international agencies. This means that environmental factors, especially socioeconomic ones, have a major impact on the transmission and severity of these diseases. Now, although they have been given top priority, these diseases are not the only ones widely affecting poverty-stricken areas. Human African trypanosomiasis kills much more today than it did at the end of the colonial era in the early 1960s. This is directly imputable to the sociopolitical upheavals that sadden Africa and disorganize the health systems of many African countries. In Latin America, Chagas disease is caused by a parasitic protozoan, *Trypanosoma cruzi*, transmitted by true bugs (triatomine bugs), that proliferate in poor habitats (Figures 3 and 4). For this reason, Chagas disease has been called a **disease of underdevelopment** [4].

These examples illustrate the fact that controlling infectious diseases is not only a matter of advanced biomedical research. **Human sciences** (sociology, anthropology, economic and political studies) are clearly indispensable to addressing the question and to understanding how local populations perceive these diseases and their treatments. This is the reason why several chapters in this book deal with human sciences.

A utopian vision for controlling emerging and reemerging infectious diseases: a world chain of large multidisciplinary research and control centers (the world CDC belt).

Utopia: what has not happened yet (the author).

Infectious diseases are not the only scourge that plunges mankind into mourning. Malnutrition, cancer, and cardiovascular diseases are also fearsome curses. However, infectious diseases still are by far the main factor of mortality. Moreover, they have a very important specificity: by nature they are **transmissible**, which is not the case for the other curses mentioned above. This particularity calls for specific means of



(*a*) (*b*)

Fig. 3. (a) *Trypanosoma cruzi*, the parasitic protozoan that causes Chagas disease. (b) a family of triatomine bugs, the vectors of Chagas disease. From left to right: male (with its proboscis, the part of the mouth for sucking blood, unfolded); female; larvae at different development stages. The whole family is obligatorily blood feeding, from the egg stage to death.

Fig. 4. The author collecting triatomine bugs, the vector of Chagas disease, in a Bolivian village. The houses are built with adobe (dried mud) and have no plaster on the walls. The bugs hide in the cracks and holes of the walls and come out at night to feed on humans and domestic animals. Improving habitats is an efficient measure toward controlling Chagas disease transmission.

control. **Infectious diseases do not stop at political borders** [6]. This means that their control should be highly coordinated among all countries of the world. The recent outbreaks of SARS and even more, bird flu, have illustrated that an epidemic that was born on the other side of the planet can reach the Western world in a matter of weeks or even days if the pathogen was brought by air transportation. However, politicians are slow in reacting to these threats [2] and the reality is that there is no world system worthy of the name for controlling the ERID peril. The efforts of WHO (*http://www.who.int/en/*) toward better international coordination deserve to be emphasized, as well as the welcome initiative of the European Union to establish a European Centre for Disease Control (*http://www.ecdc.eu.int/*). However, we are still a far cry from a truly integrated world system, something like the US Centers for Disease Control, or more exactly, its branch specialized in infectious diseases, the National Center for Infectious Diseases (*http://www.cdc.gov/ncidod/*), but **on the scale of the entire planet**. The NCID is a large federal organization that is staffed with roughly 2,000 people. Its originality is its three-fold mission: research, specialized training, and surveillance/control. I have long proposed [6,7] creating a centralized structure in Europe patterned after the US CDC, with a comparable size and a similar triple function. Indeed, the ECDC cannot be compared with the US CDC. It is a small organization with only 60 staff members, devoted to surveillance only. Although a valuable first step, it could be dramatically insufficient in the advent of a major pandemic [10].

It is worth noting that the strongest initiative in this domain is coming from a region of the world that is far less wealthy than the great Europe of 25: ASEAN (Association of Southeast Asian Nations; *http://www.aseansec.org/*), groups together Brunei, Burma, Cambodia, Indonesia, Laos, Malaysia, the Philippines, Singapore, Thailand, and Vietnam. These ten countries plan to group their resources to set up a large structure comparable to the US CDC, the ASEAN CDC [11].

Actually, the world needs **a warning belt** of comparable structures throughout the world, structures that are highly integrated and interconnected, with clearly defined task distribution and geographical responsibility. This belt should comprise the existing **US CDC**, plus the **ASEAN CDC** that should include Asian countries outside the ASEAN (China, India, Korea, Japan, and others), a considerably enlarged version of the **European CDC**, extended to Turkey and the former USSR [6,7,10,11]. A comparable center in **South America** as well as one in **Africa** should complete the system. An **African CDC** is even more desirable, given that this region of the world is suffering more than others from the curse of epidemics. Large centers of this type, with advanced research and information systems, especially those that would be located in southern countries, would create many jobs with considerable expertise, would highly stimulate local research, and would fuel local surveillance and control systems. This would help counter the distressing brain drain of young talents from the south toward industrial countries.

These centers should not limit themselves to classical biomedical research, but should rather strongly engage themselves in groundbreaking, multidisciplinary research, sorely needed, as exposed above. A difficult equilibrium between hard versus human sciences, high-tech versus traditional know-how, and bench vs field studies should be fostered in this **world CDC belt** [12]. At present, this is being done nowhere in the world.

The Encyclopedia: A First Handbook for Multidisciplinary, Holistic Research in Infectious Diseases. Its Authors: An Ideal Pilot Team for Research Centers of the Future.

When I designed the content of this book, I had in mind this need for a definite multidisciplinary approach to the ERID peril. The eminent colleagues I have selected as contributors, many of whom are close friends of mine, could be considered as **a pilot team for a future research and control center**, a kind of "dream CDC" of the sort that should compose the **world CDC belt**. The collection of disciplines and expertise brought together in this book is impressive. It is not exhaustive, but provides the reader with many of the aspects of our present efforts toward better knowledge and control of epidemics. The spectrum of topics includes the more advanced technologies (satellites in Chapters 23 and 26; advanced molecular biology and genomics in many chapters. As humorously noted by a colleague of mine: the encyclopedia goes "**from microsatellites to satellites**"). The magic power of these new technologies is especially illustrated by Chapters 4 (characterization of uncultivable pathogens) and 27 (fossil DNA of mummies).

The three diseases of poverty (malaria, tuberculosis, AIDS) are given special, but not exclusive attention.

Chapters 2 and 7 illustrate the fact that the problems raised by epidemics and infectious diseases are comparable where pathogens of **either human or animals or plants** are concerned.

This book is not written only for people involved in medical sciences. It has also been designed to be of interest to veterinarians and agronomists. Human and animal pathogens are frequently the same. For example, the agent of Chagas disease can infect any mammal, not to mention the recent, dramatic example of bird flu. Studying animal pathogens is therefore crucial for human health, besides the obvious economic relevance of it.

Ample space is devoted to **evolutionary studies**, which provide us with many keys for understanding infectious disease transmission and which show the usefulness of basic research in medical applications, and in turn, the contribution of the study of infectious diseases to fundamental biological knowledge.

The need to address **a global scale** and provide a bird's eye view of the problem is emphasized by Chapters 32 and 33.

The heuristic value of **integrated studies including host, pathogen, and vector** in the case of vector-borne diseases [8] is shown by Chapters 6 and 16.

Practical **field experience** is brilliantly exposed in Chapter 37. Chapter 24 shows the relevance of its thorough use in a sophisticated, international network surveillance.

The threat of **bioterrorism** is exposed by a CDC expert of the field in Chapter 36.

Chapters dealing with the **human science** side of the question (neglected diseases, the points of view of sociology, anthropology, history, and economics) give us another perspective, indispensable to evaluate the **perception** of these curses and the impact of sociocultural and political problems on the transmission and severity of these diseases.

Last, the ending chapter shows us how much epidemics in the past have struck the imagination of mankind and have inspired (and still do?) fear on a religious scale.

READERS TARGETED BY THE ENCYCLOPEDIA

This book addresses a broad and varied audience. The authors have been selected among top professionals in their field. The chapters are therefore a reflection of the most up-to-date science and can be used as reference for specialized scientists. However, the contributors have been warmly invited to make their chapters accessible to a broad audience beyond the specialists of their field. To help in reaching this goal, **detailed glossaries of specialized terms** accompany most chapters. Of course many terms are defined several times in different glossaries. I chose not to establish a common glossary because some terms may have slightly different definitions according to the background and the vision of the authors. This is enriching and shows that science is not written in stone. **Simplified classifications of pathogens** (viruses, parasites, and bacteria) are given in Chapters 9, 18, and 37, respectively. Thanks to this effort toward accessiblity, many non-research-oriented readers can benefit from this book, including medical doctors, veterinarians, all health professionals, agronomical engineers and technicians, professors, teachers, students, and the educated public.

My dearest wish is that this book will arouse many vocations among its youngest readers, some of whom could some day staff the **world CDC belt**.

ACKNOWLEDGMENTS

My thanks are extended to our Wiley editors, who accepted this very atypical and therefore risky project. I thank all my contributors for their eminent contributions that will make it successful. My special thoughts go to my eminent collaborator and friend Francisco J. Ayala, and to my dear collaborators of my Montpellier laboratory, who made possible the long-term research that is behind Chapters 19 and 20. Lastly, I thank all the friendly people and colleagues from the various countries I have stayed in, who have enriched me with their culture and expertise, and have made my professional life a constant delight.

IRD representative office in Bangkok, March 2006.

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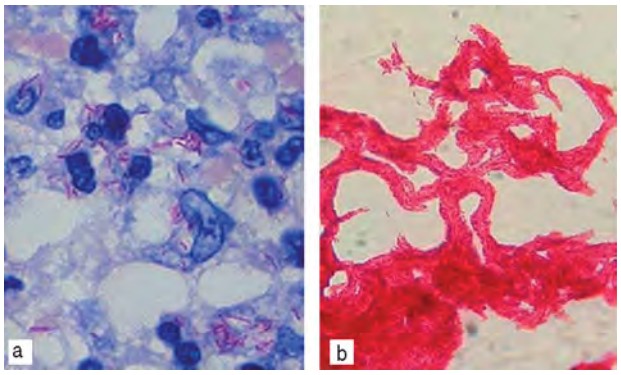


Fig. 1.2. *Mycobacterium tuberculosis* (MTB): *Ziehl–Neelsen* coloration See text for full caption.

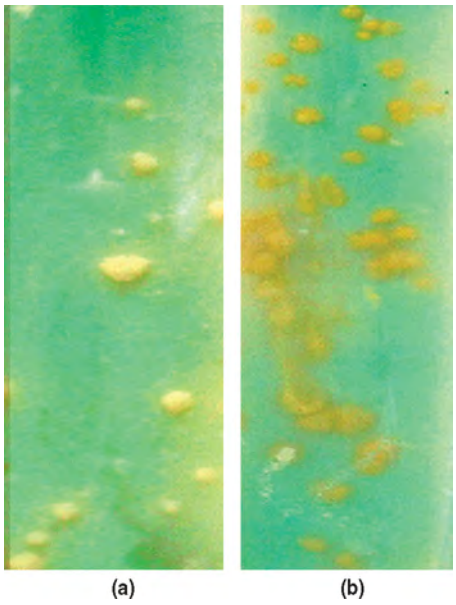


Fig. 1.4. Colonies of *Mycobacterium* on Lowenstein–Jensen. See text for full caption.

Fig. 2.1. (a) Glanderous horse (photo T. Morton / U.Wernery, 2004)

Fig. 2.2. Rinderpest in an African calf (FAO/OIE document).

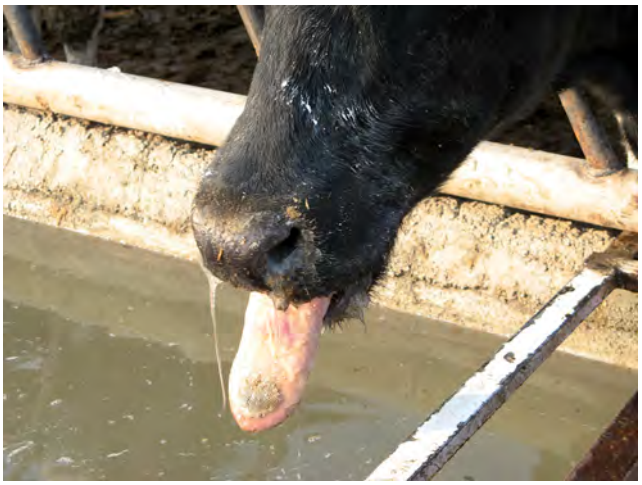


Fig. 2.4. Foot and mouth disease: lesions on the tongue (FAO document).

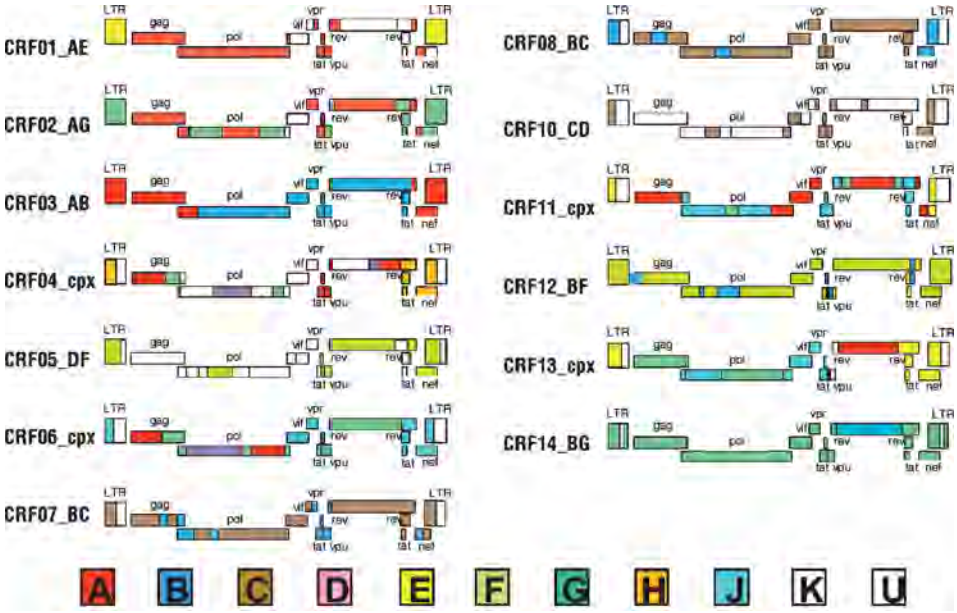


Fig.3.4. Mosaic genome of different Circulating Recombinant Forms (CRFs).

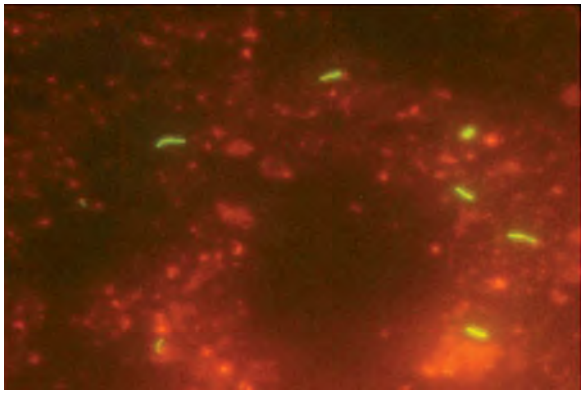


Fig. 5.1. Auramine staining of a sputum smear: we can notice the presence of acid fast bacilli, fluorescent, on a red background of eukaryotic cells.

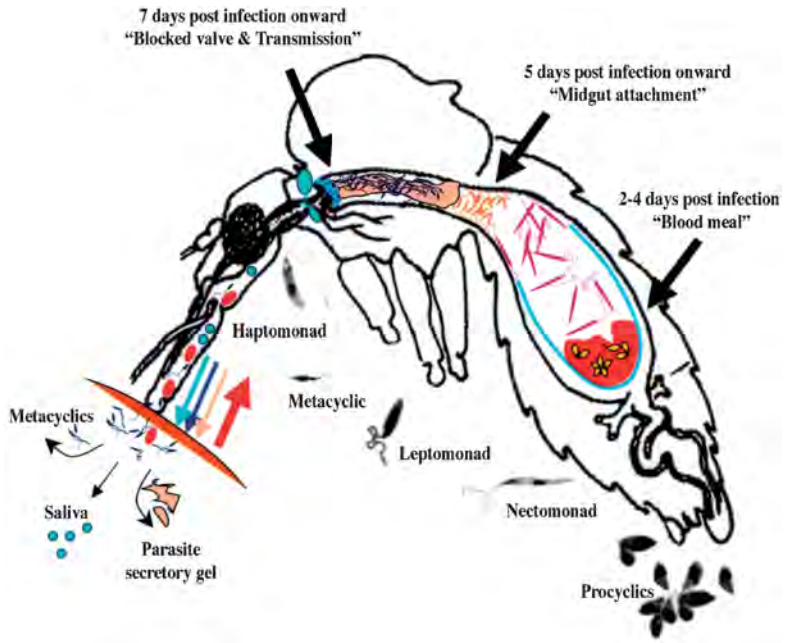


Fig. 6.10. Life cycle of *Leishmania* in a competent sand fly vector.

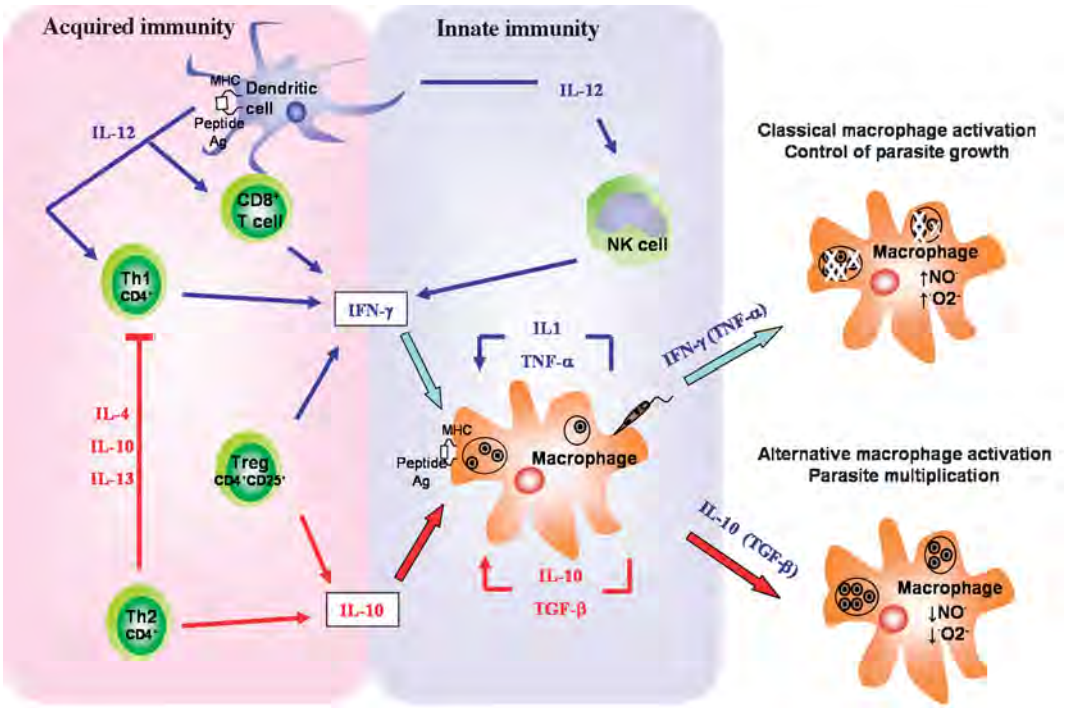


Fig. 6.12. Immunological determinants influencing parasite multiplication. See text for full caption.

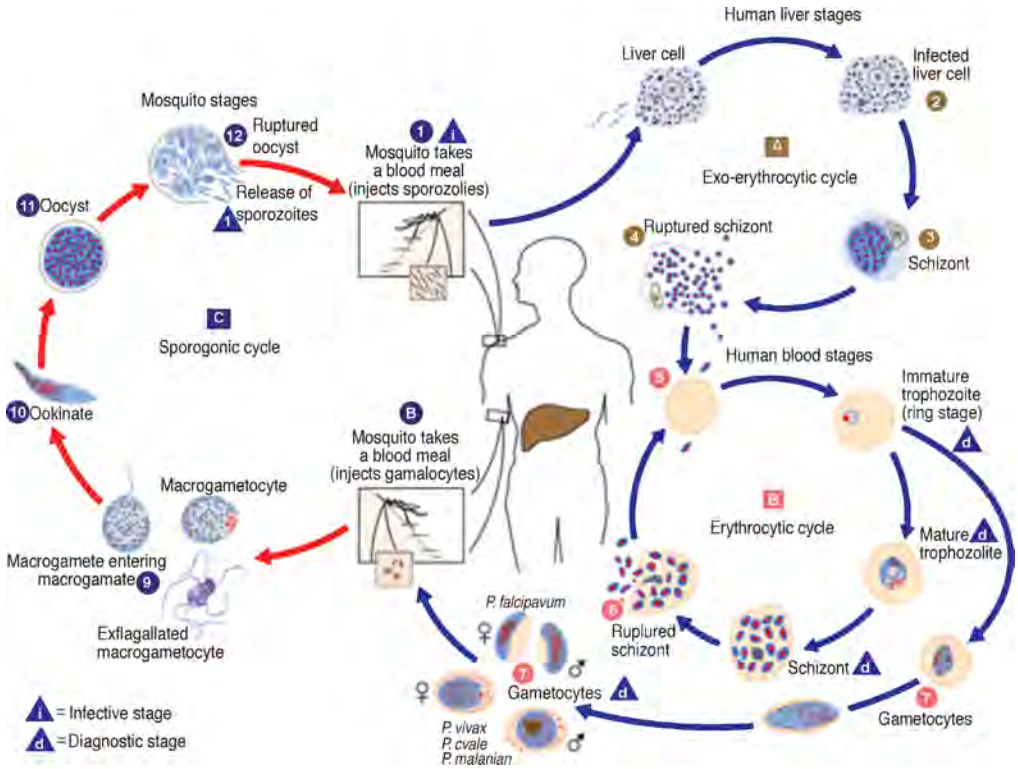


Fig.8.2. The schema of the life cycle of malaria. See text for full caption.

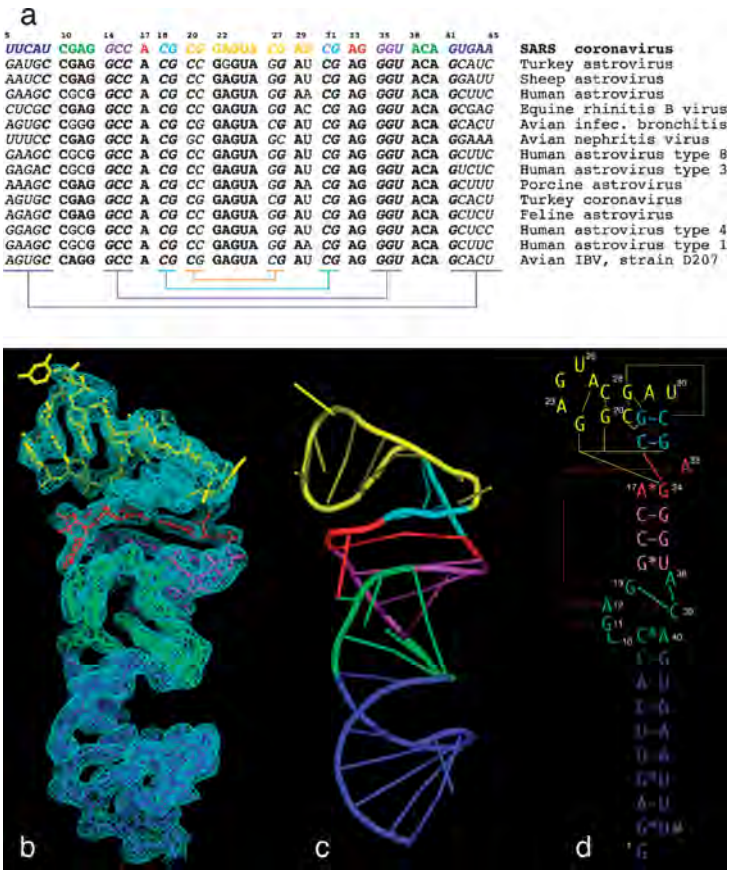


Fig. 9.3. (reprinted from [54]). The primary, secondary, and tertiary structures of the SARS s2m RNA. (A) Phylogenetic comparisons of RNA sequences from various viruses. The SARS RNA sequence is color coded to match the color scheme used throughout. Conserved sequences are highlighted as bold letters, and co-varying sequences involved in conventional RNA helical base pairing are indicated in italics. Sequence complements are indicated using color-coded brackets. (B) Experimental electron density map contour that allowed unambiguous tracing of the RNA molecule. (C) A corresponding ribbon diagram highlighting the unusual fold. (D) Schematic representation of the RNA secondary structure with tertiary structural interactions indicated as long-range contacts.

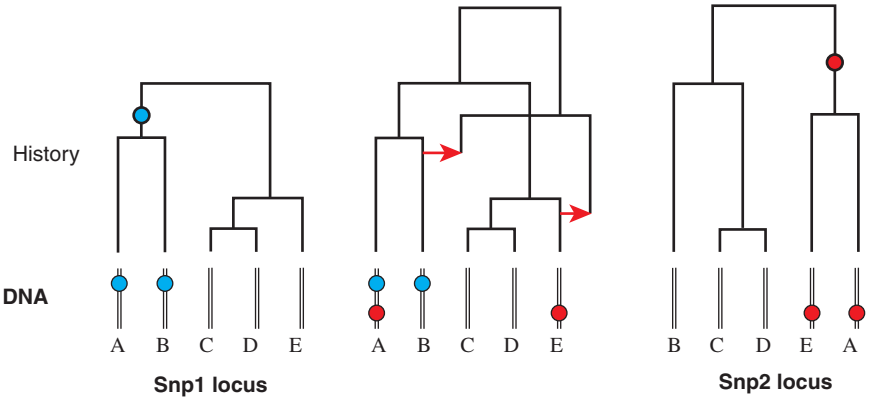
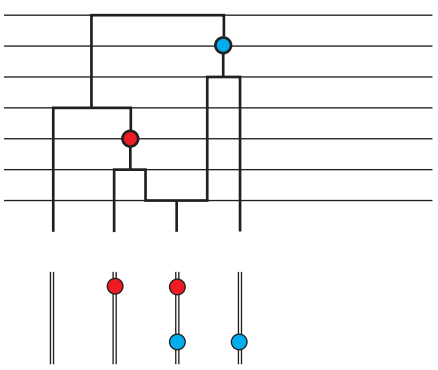


Fig.10.1. The effect of recombination on gene genealogies at physically linked sites. Recombination allows for individual segregating sites, or SNPs as are shown here, to have independent evolutionary histories. The history in the middle is an ancestral recombination graph integrating the two histories.



Event	Probability
Coalescence	$1 / (\rho + 1 + \theta)$
Mutation	$\theta / (4\rho + 4 + 4\theta)$
Coalescence	$1 / (\rho + 3 + 5\theta/4)$
Coalescence	$1 / (3\rho/2 + 6 + 7\theta/4)$
Mutation	$\theta / (6\rho + 24 + 7\theta/4)$
Coalescence	$1 / (3\rho/2 + 10 + 2\theta)$
Recombination	$\rho / (4\rho + 10 + 4\theta)$

$\theta = 4N_e\mu$ $\rho = 4N_e r$

Fig.10.3. Coalescent modeling of recombination. The coalescent is a "backwards" process for modeling genealogies. Parameters of critical interest, such as mutation and recombination, can be mapped onto these genealogies. In this way, we can see how both the genealogical history and recombination affect the pattern of polymorphism among sites in the genome.

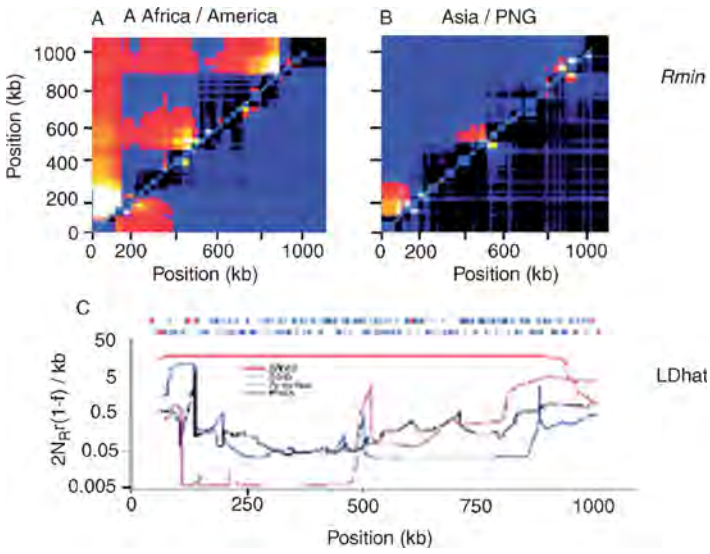


Fig.10.4. Hotspots of recombination in four populations of *P. falciparum* [75]. The two top panels are estimates of the minimum number of recombination events, for each of four different populations, for all pairs of sites obtained using the method of Myers and Griffiths (2003) with the program Recmin. The bottom panel shows parametric estimates of population recombination rate variation across the third chromosome using the program LDhat.

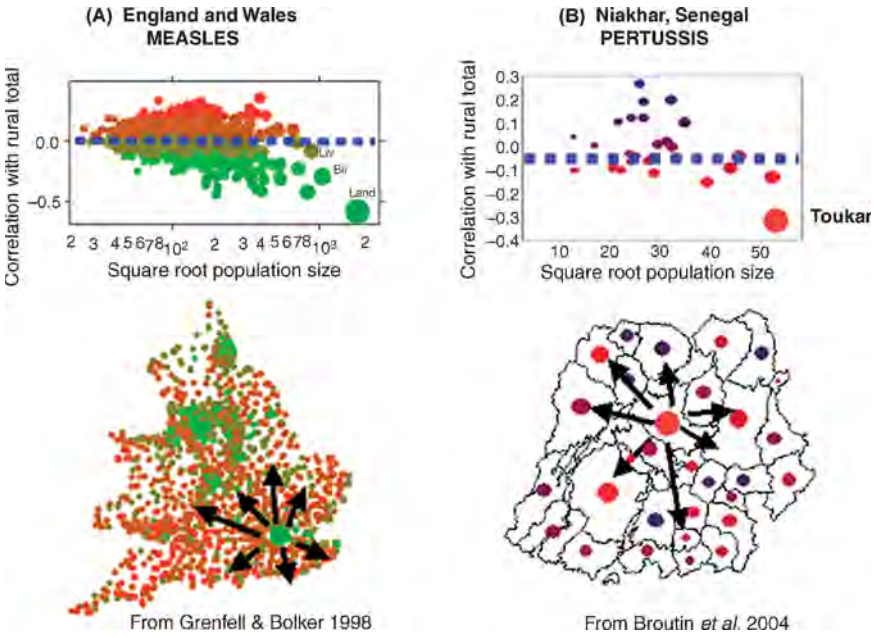


Fig. 12.7. Illustration of the comparison of infectious disease spread at two different spatial scales. The left part (A) describes the spread of measles in England and Wales (*cf.* [21]). The right part (B) shows pertussis spread in the rural area of Niakhar, in Senegal (cf. [8,9]). In the latter, orange (respectively, green) denotes negative correlations between measles in a population (as proportion of total cases) and measles in a "rural" aggregate (see text for details). For each part, the top graph provides on the *y*-axis the correlation between the "rural" aggregate and the time series in individual populations (845 localities in A and 30 in B), in relation with the population size (on the *x*-axis). A negative correlation describes a delay between rural cases and epidemic increase in the study population. The bottom maps are a spatial representation of the upper graphs. More analyses (not shown here) confirm that cases appeared first in the biggest localities and then in rural populations (black arrows symbolize disease diffusion).

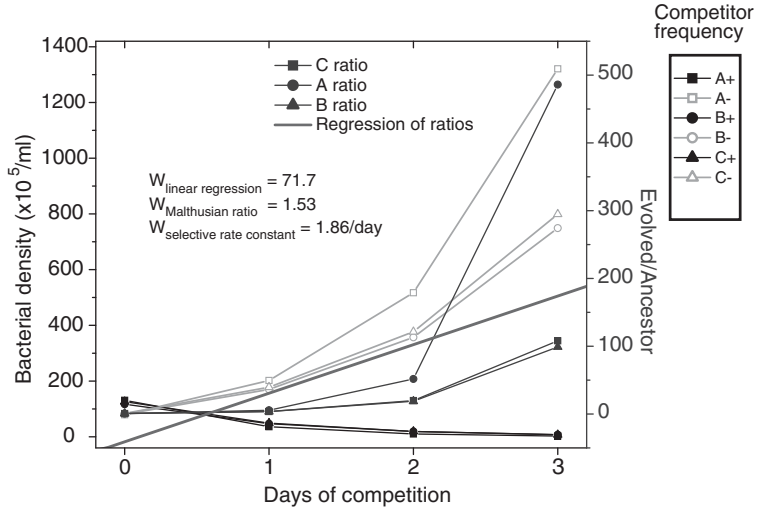


Fig.14.2. Competition between evolved and ancestral genotypes derived from a 20,000 generation evolution experiment involving *E. coli* growing in a simple salts medium with glucose as the sole carbon source [3,16,17]. Relative frequencies of the ancestor and evolved genotypes are plotted as raw counts (left ordinate) and as ratios (right ordinate) over time. Fitness of the evolved genotypes is calculated relative to the ancestor by each of the three different methods described in the text. W = fitness.

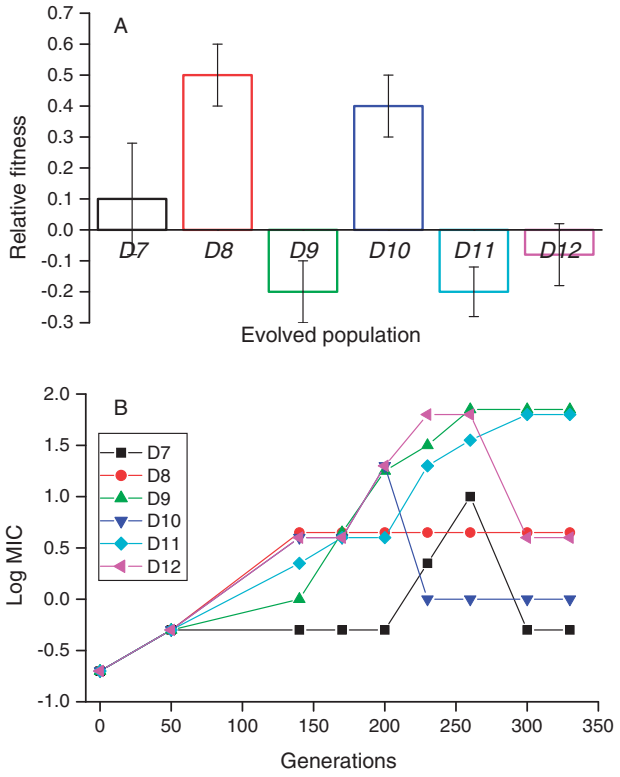


Fig.14.7. Relative fitness (A) and evolved level of fluconazole resistance (B) of six populations of *C. albicans* propagated for 330 generations in the presence of increasing levels of the antimicrobial drug fluconazole. Here, fitness was quantified in a medium lacking fluconazole and estimated as the difference in the numbers of doublings between the evolved population and the genetically marked progenitor, standardized by the total number of doublings in the assay, which varied across environments. The six populations achieved approximately three different levels of fitness relative to the ancestor. Some of the variation within populations in fluconazole resistance over time resulted from fluctuations in different genotypes that coexisted in the experimental environment. Note that the less fit populations are among the most resistant to fluconazole. From Cowen et al. [6].

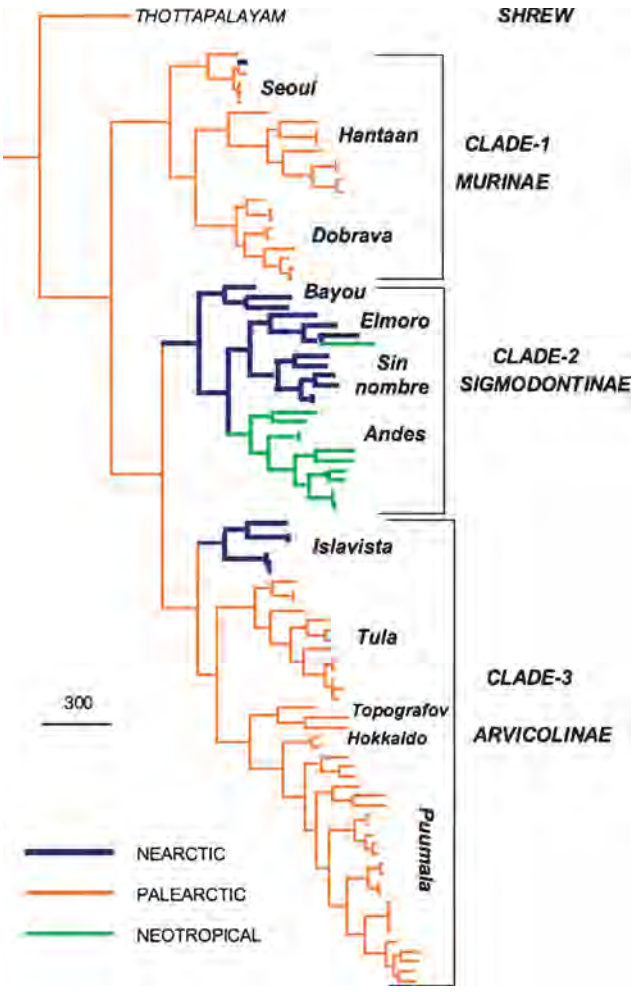


Fig. 16.8. Phylogram resulting from Bayesian analysis using GTR + I + G model. Different color patterns are attributed to different biogeographical areas. Three main clades may be recognized. CLADE-1 and CLADE-2 are detailed in Figure 16.9. CLADE-3 is detailed in Figure 16.10.

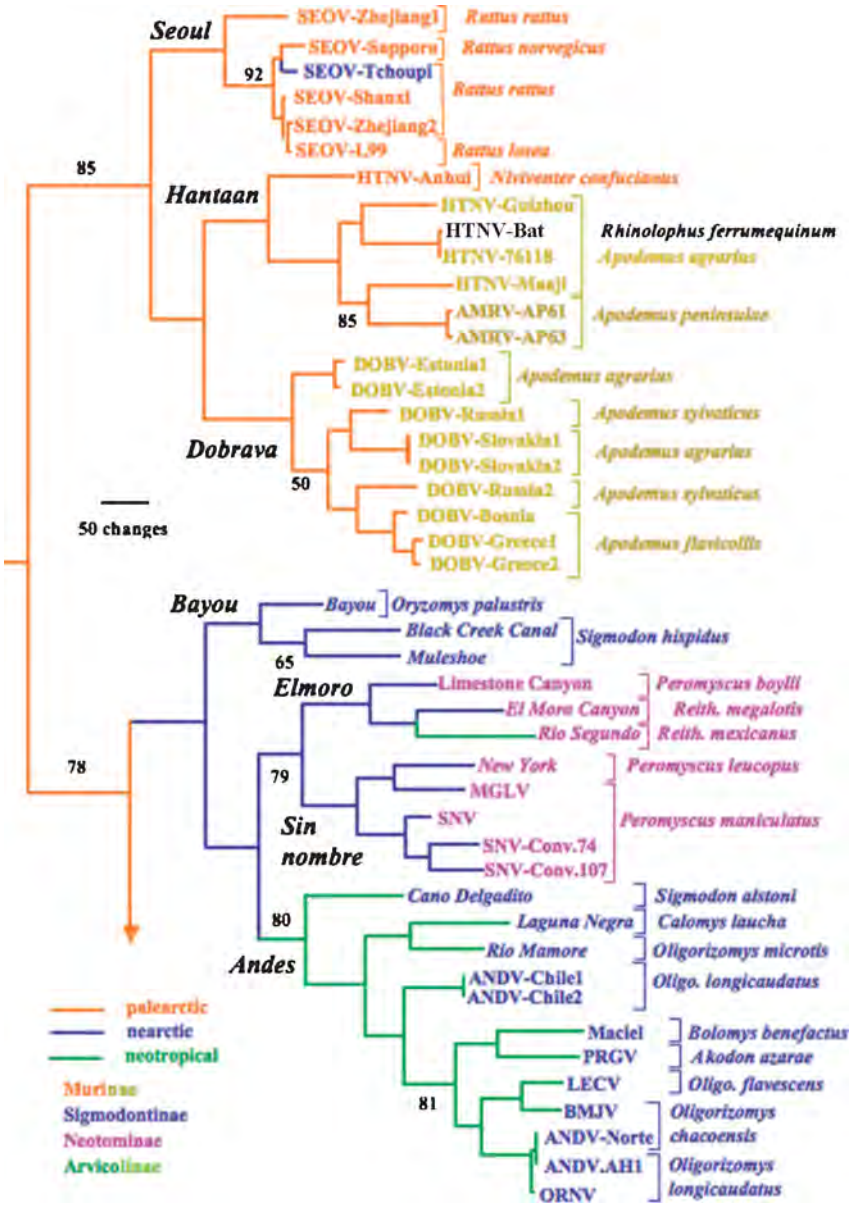


Fig. 16.9. Detail of CLADE-1 and CLADE-2 of Figure 16.8. Posterior probability numbered when inferior to 95% (probability of no numbered nodes between 95 and 100). The scientific name of host for each virus strain is given; different color patterns are attributed to different host groups and to different biogeographical areas. Reith., Reithrodontomys; Oligo., Oligorizomys.

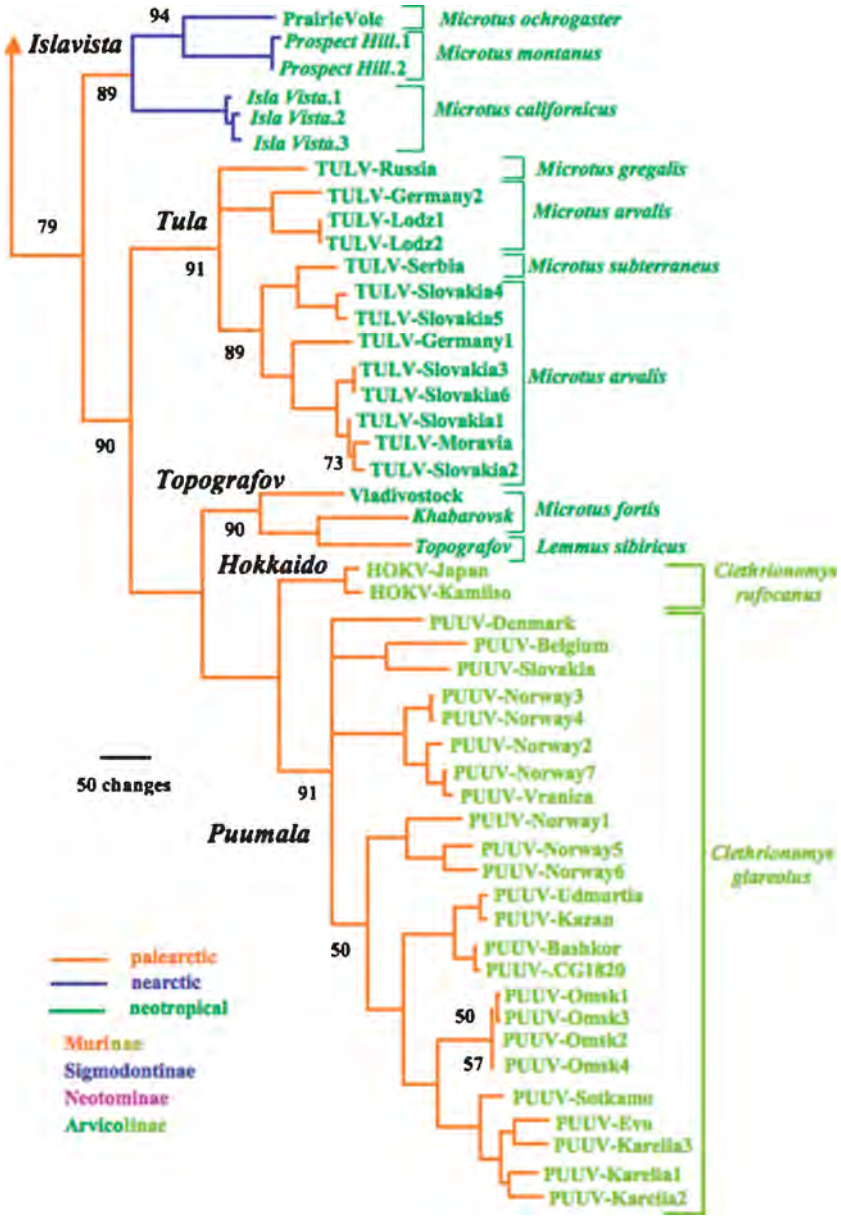


Fig. 16.10. Detail of CLADE-3 given in Figure 16.8. Posterior probability numbered when inferior to 95% (probability of no numbered nodes between 95 and 100). For each virus strain, the scientific name of host is given; different color patterns are attributed to different host groups and to different biogeographical areas.

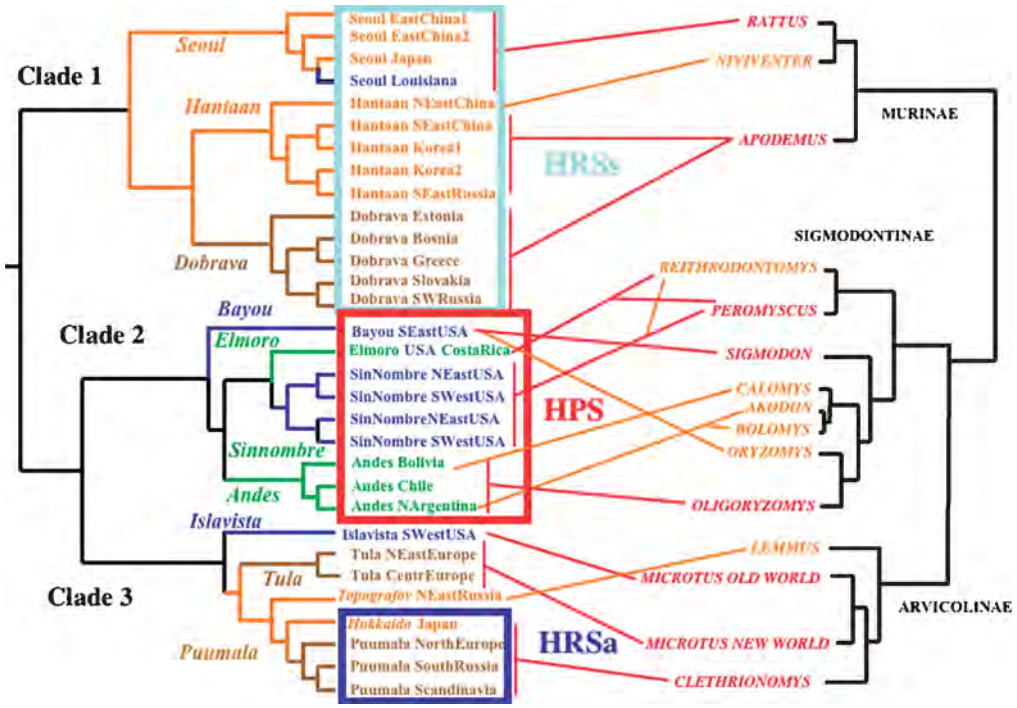
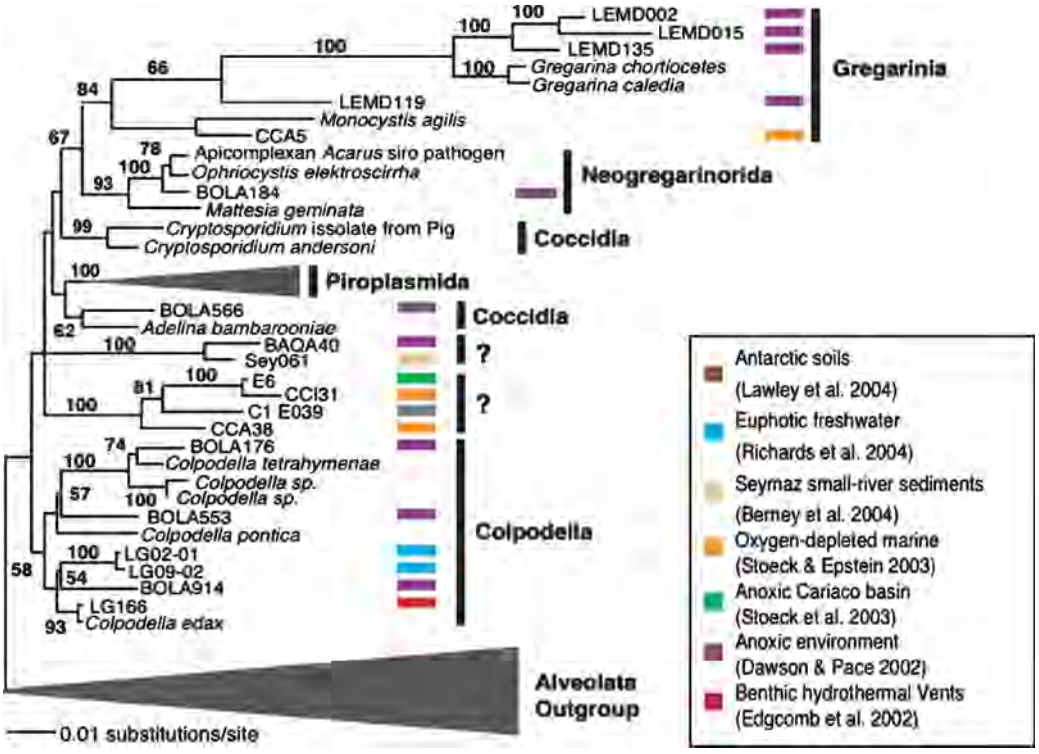
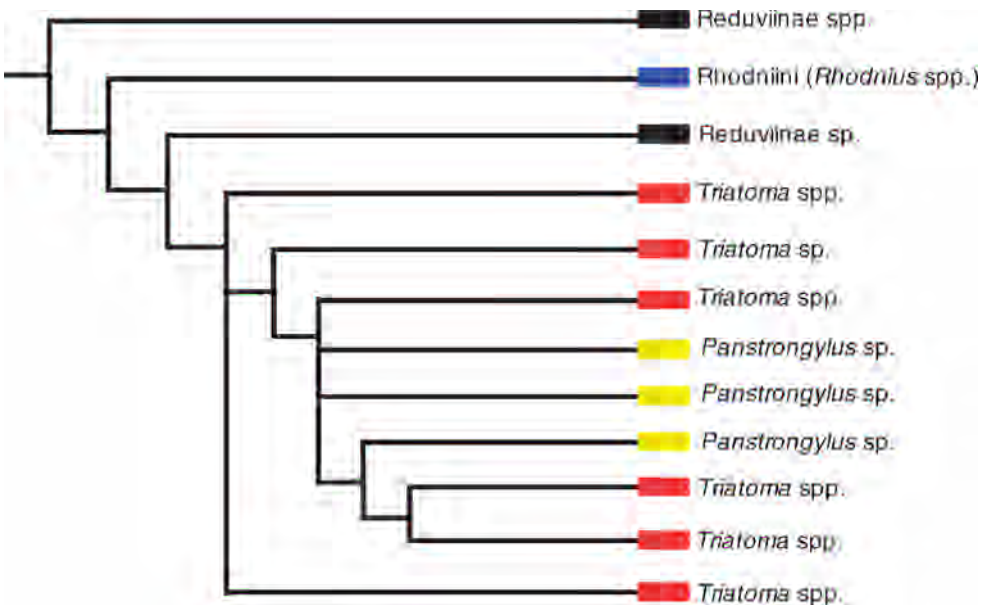


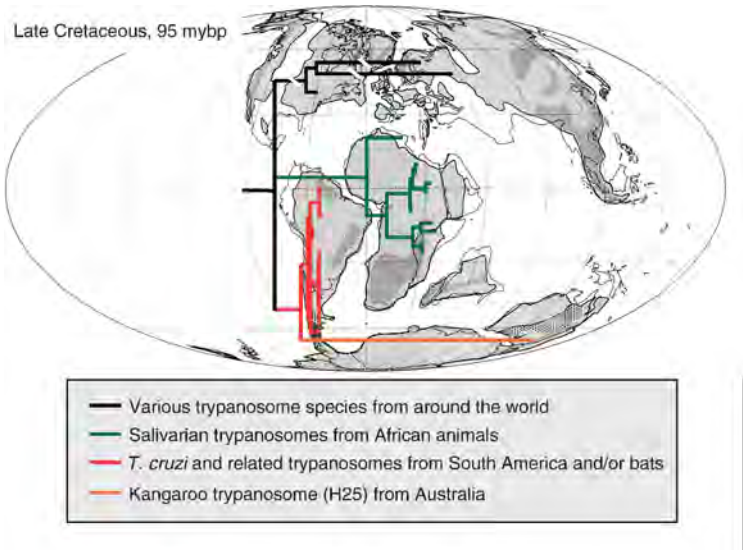
Fig. 16.11. Correspondence between the phylogeny of genus Hantavirus, the classification of its hosts, and the type of human syndrome.



Box Fig. 17.3



Box Fig. 17.5



Box Fig. 17.7

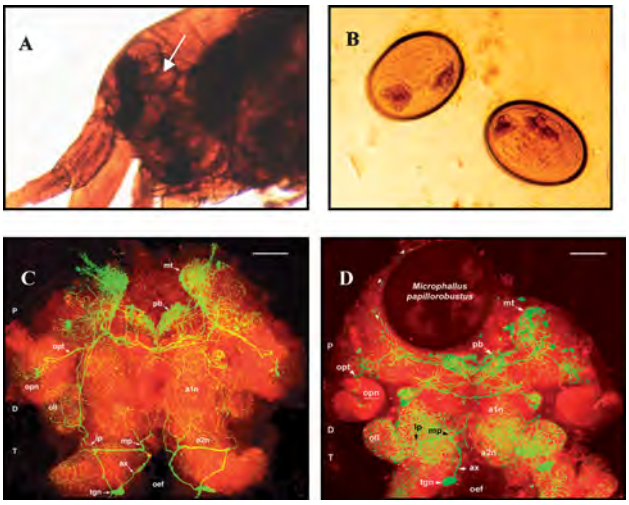


Fig. 18.2. *Gammarus insensibilis* infected by the trematode *Microphallus papillorobustus*. See text for full caption.

Fig. 18.4. Infection with the trematode *Podocotyloides stenometra* causes coral polyps to become swollen and pink (photo: Greta Aeby).

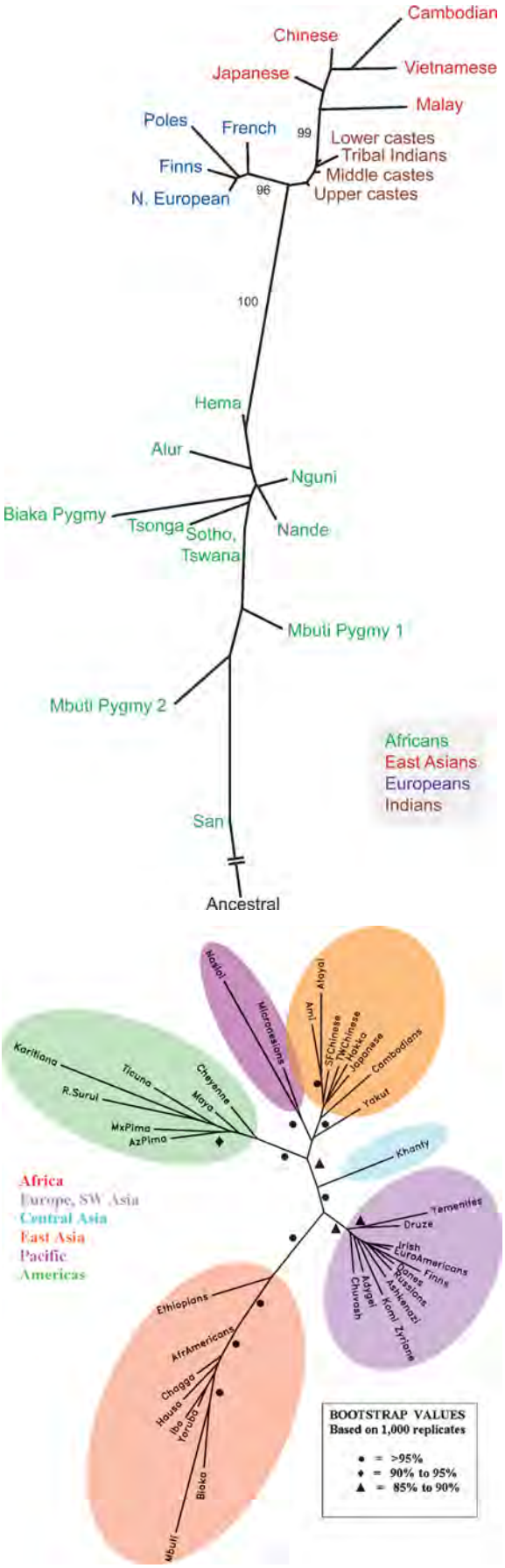


Fig. 19.4. Phylogenetic trees based on modern molecular markers. See text for full caption.

(a)

(b)

(c)

Fig. 19.6. Aymaras (a) and Quechua (b) Indian people in Bolivia. See text for full caption.

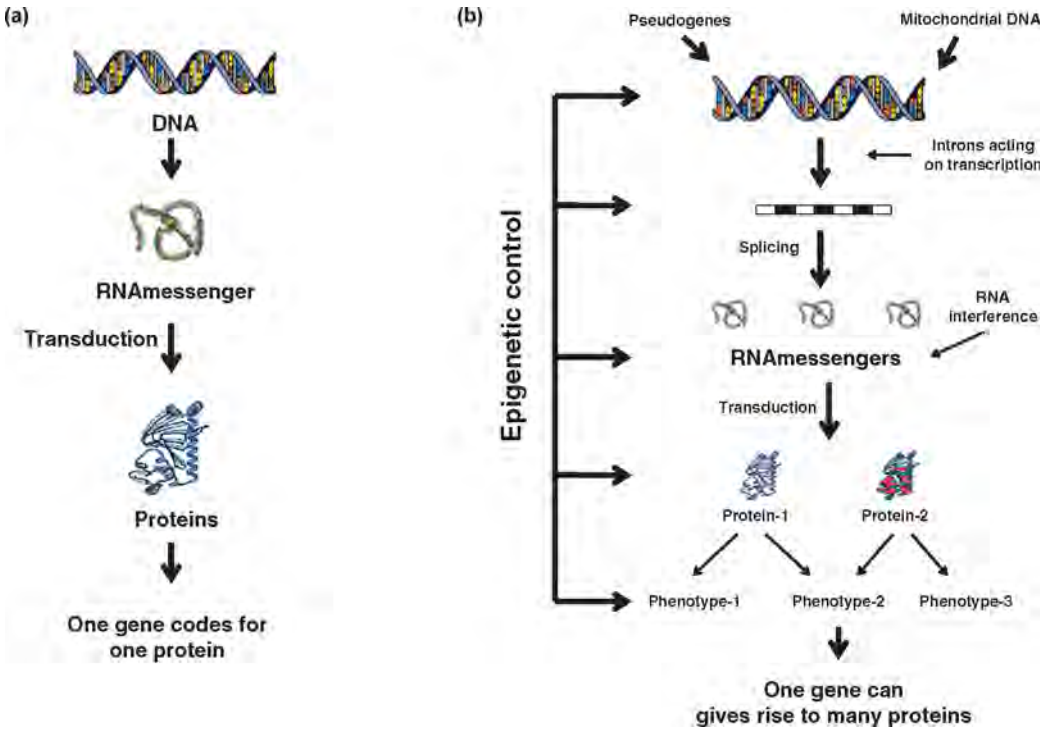


Fig. 21.1. The central dogma of molecular biology of the genomic era (a) and of the post-genomic era (b).

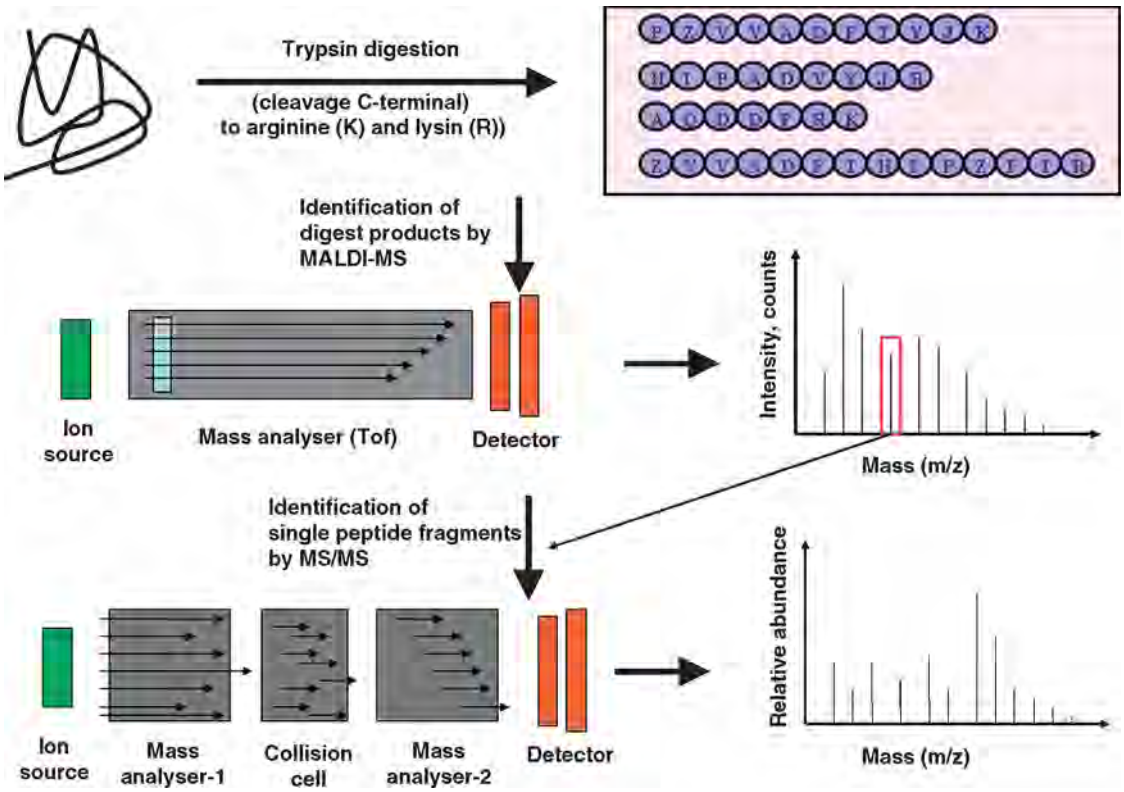


Fig. 21.2. Schematic illustration of the identification of protein by MALDI-TOF MS (mass spectrometry) and by tandem MS.

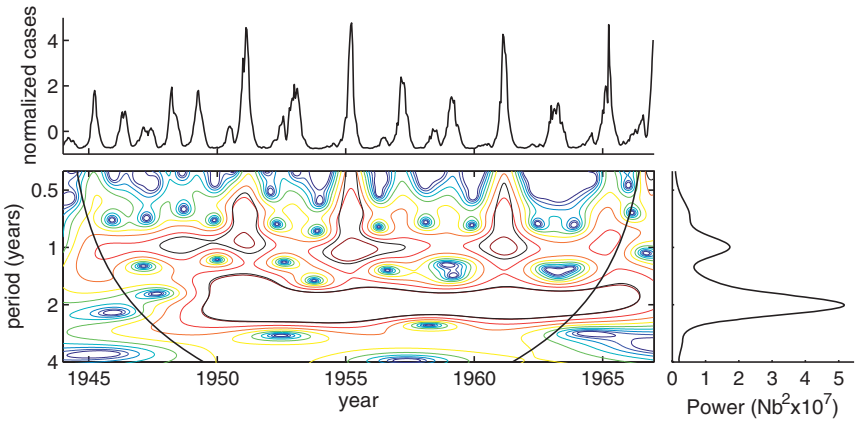


Fig. 22.12. Wavelet power spectrum of the measles notification cases for London between 1944 and 1966. See text for full caption.

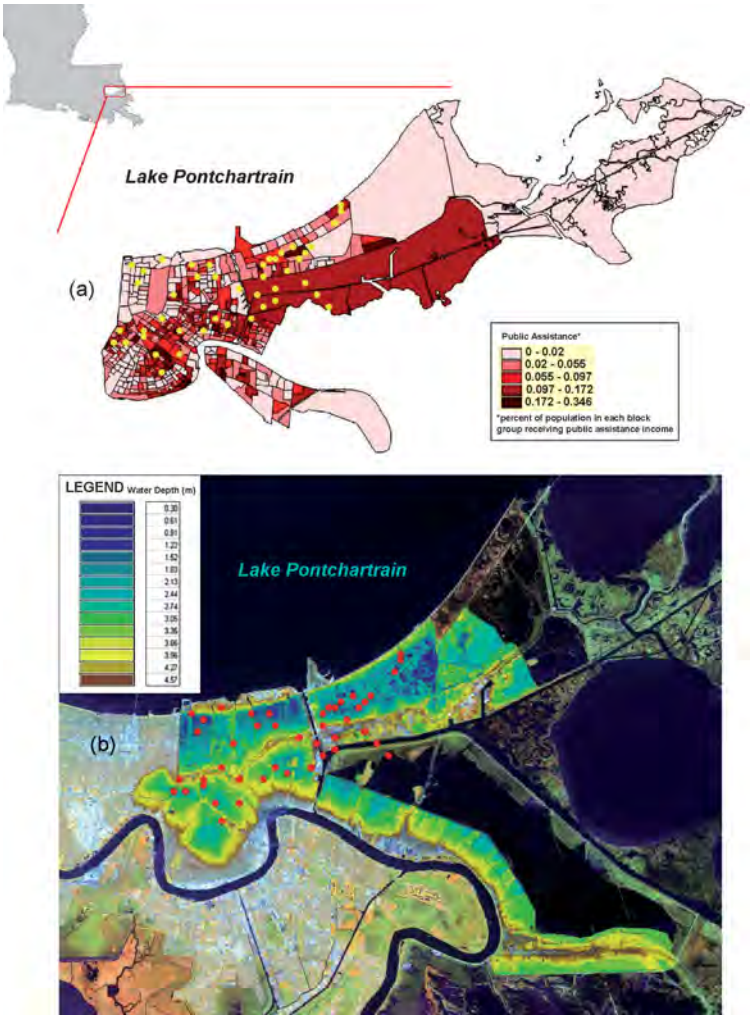


Fig. 23.1. (a) The distribution of deaths related to Hurricane Katrina for the city of New Orleans overlaid onto a choropleth map of public assistance, as defined by the percentage of the population on welfare. (b) The distribution of Katrina-related deaths overlaid on a raster grid of post-levee failure floodwater depths throughout New Orleans. *Floodwater data developed by the U.S. Federal Emergency Management Agency's Joint Field Office, September 2005.*

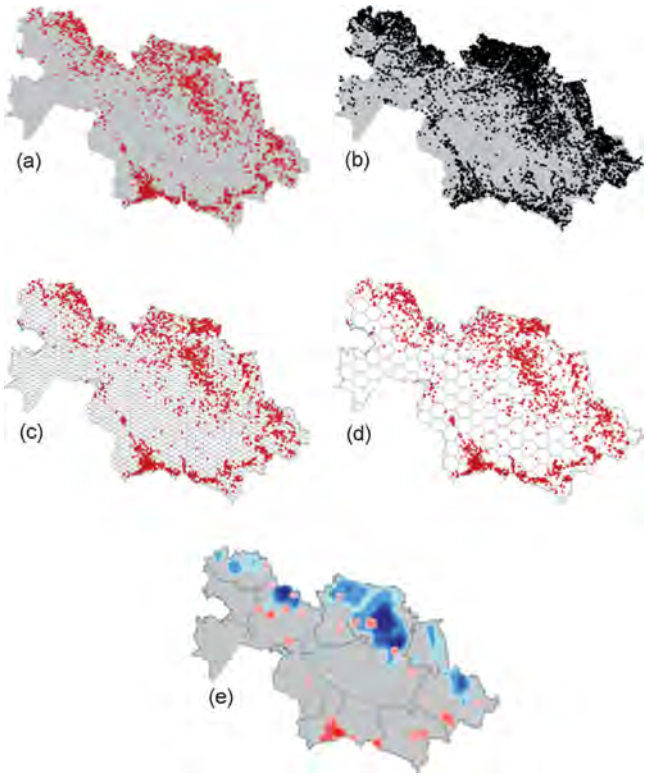


Fig. 23.3. (a) A point map representing the distribution of livestock anthrax cases for the country of Kazakhstan from 1937 to 2004. See text for full caption.

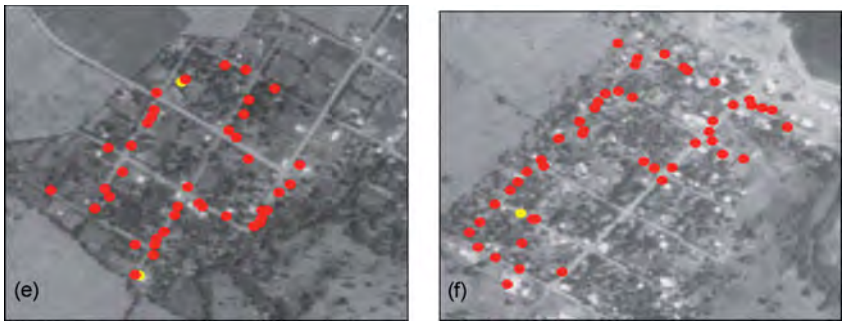
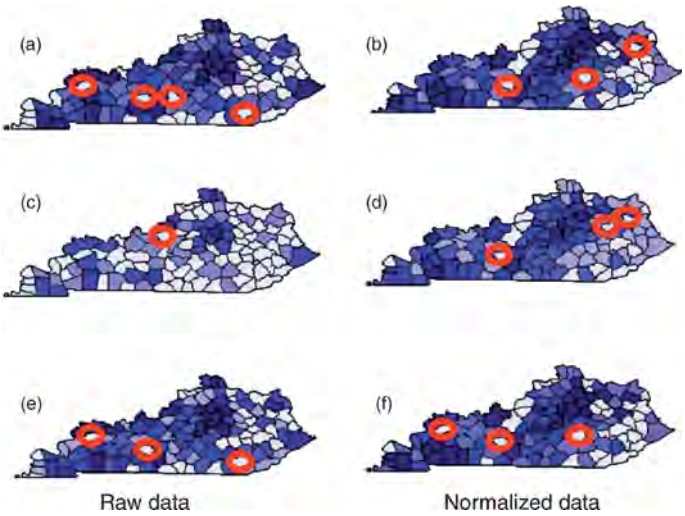


Fig. 23.6. (a) A screen capture of the LSU WHOCC web site used as a portal to the Chagas online data entry system and the Chagas map web-GIS. See text for full caption.



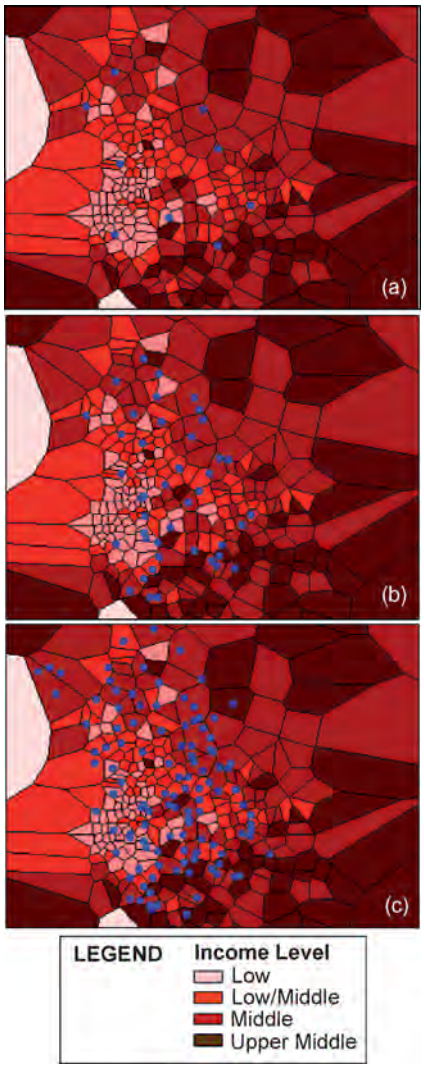


Fig. 23.4. The distribution of HIV/AIDS cases over a 10-year period. See text for full caption.

Fig. 23.9. The distribution of rabies surveillance data at the county level for the state of Kentucky using choropleth mapping comparing differences in classification techniques, the number of classes, and raw versus normalized data. See text for full caption.

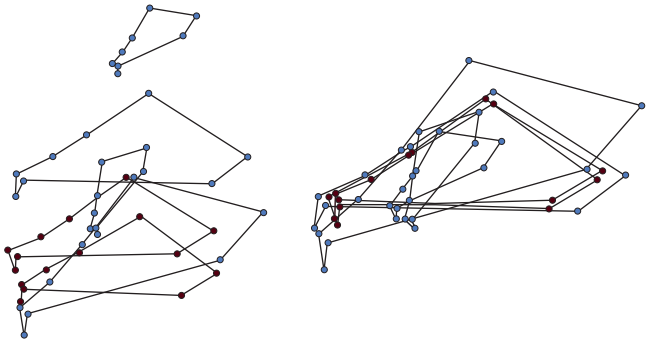


Fig. 25.5. This figure shows the effect of the translation step (left → right) for six configurations of eight wing landmarks (see Fig. 25.2) of the genus *Rhodnius* (Hemiptera, Reduviidae). See text for full caption.

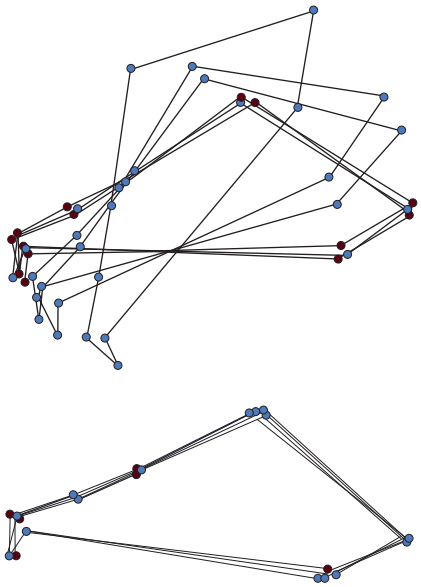


Fig. 25.6. Two more steps of the Procrustes superimpostion, the scaling (top) and the rotation (bottom) steps. See text for full caption.

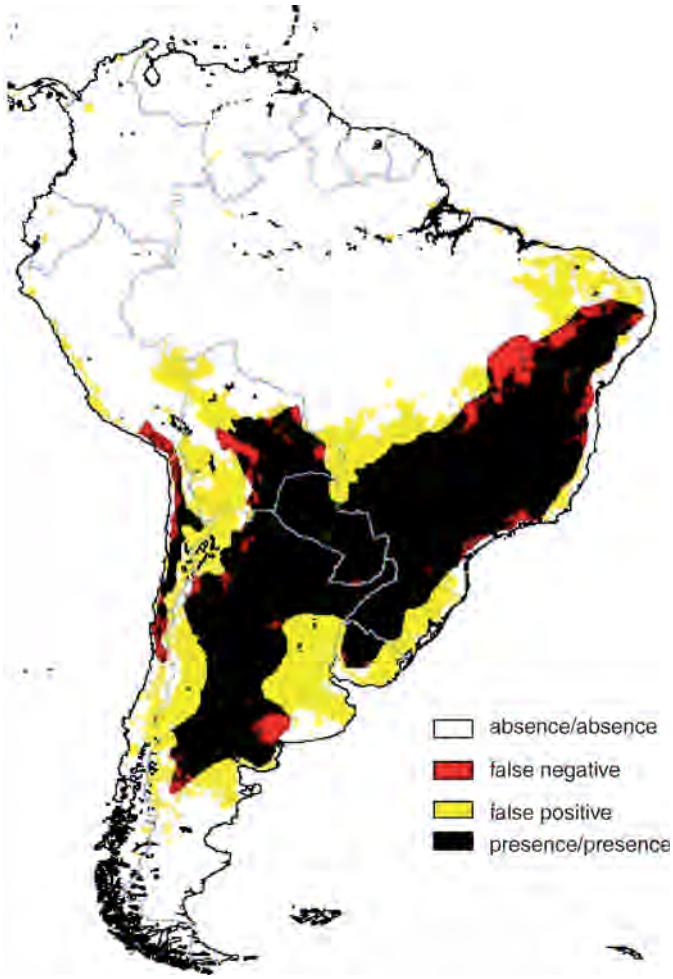
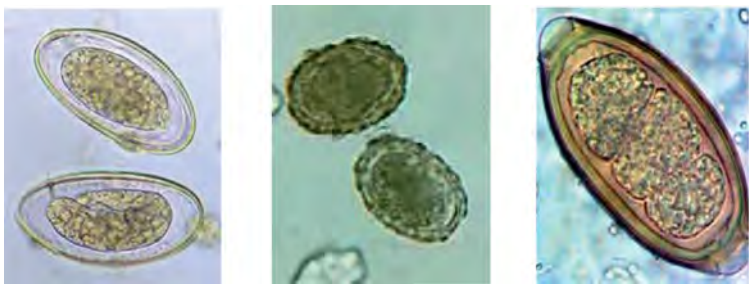
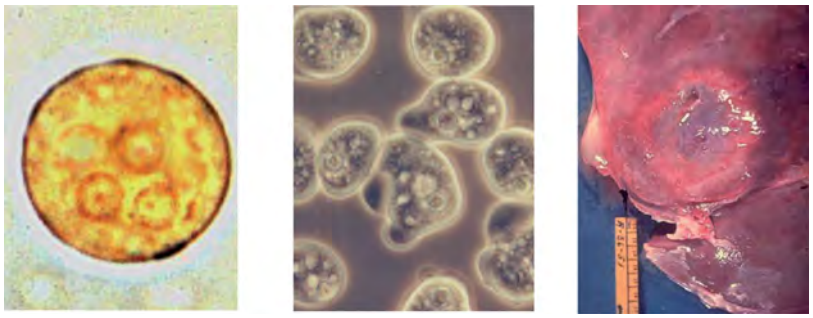


Fig. 26.5. Potential geographic distribution of *T. infestans*, estimated from a classification based on six environmental variables (four related with temperature, one with maximum middle infrared, and one with maximum of the NDVI), with the higher loadings estimated by a stepwise discriminant analysis, among 56 variables derived from a temporal Fourier analysis of the 1982–2000 time series of AVHRR images. See text for full caption.

Fig. 27.3. This heart was present in the thorax of the body in this figure. See text for full caption.



Box Fig. 27.5.



Box Fig. 27.6.

Box Fig. 27.7.

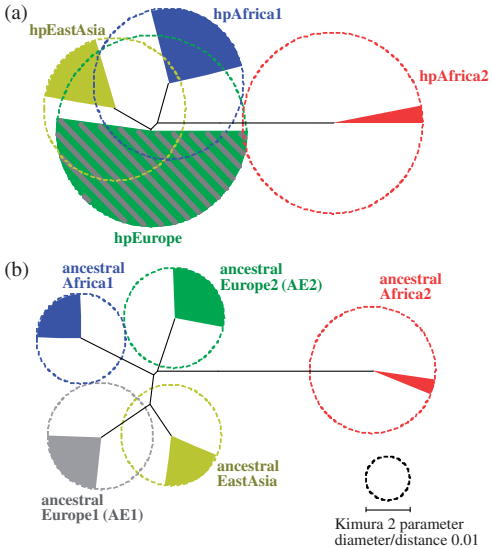


Fig. 28.10. Relationships between modern populations (a), modern subpopulations (b), and ancestral populations (c) of *H. pylori*. See text for full caption.

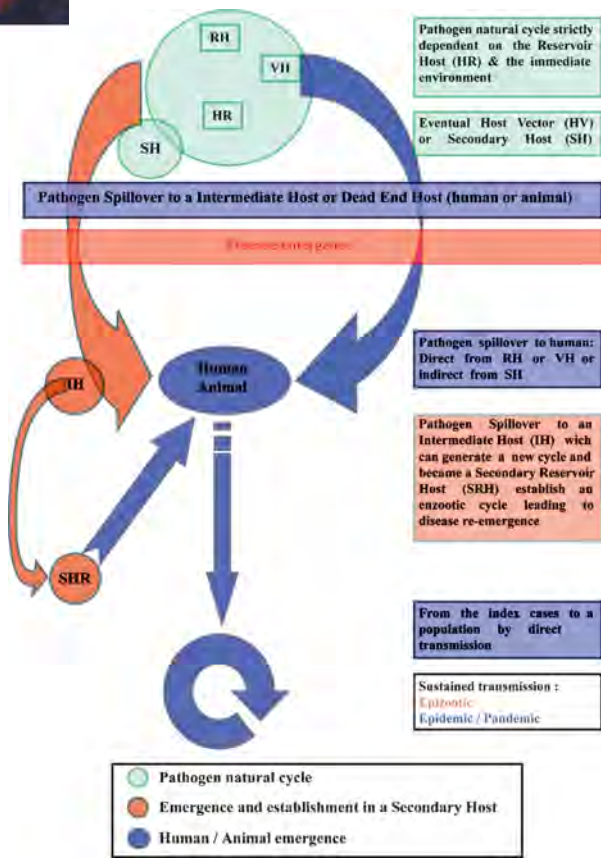


Fig. 32.1. Infectious disease emergence (a sketch): the general pathways of infectious disease emergence (adapted from childs et al., 2004, in press): See text for full caption.

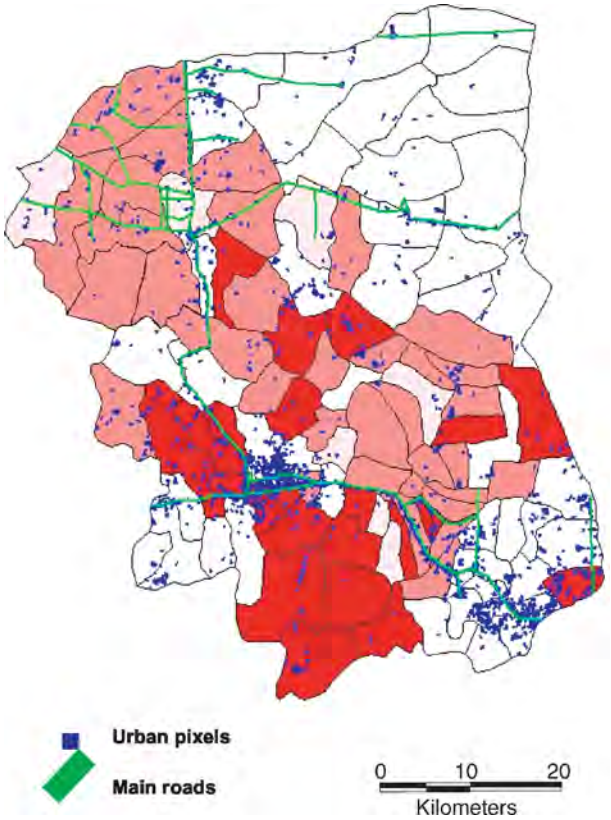


Fig. 32.6. "Urban pixels" distribution and the number of epidemic months among the 103 sub districts (1997–1998 Dengue outbreak, Nakhon Pathom Province, Thailand). See text for full caption.

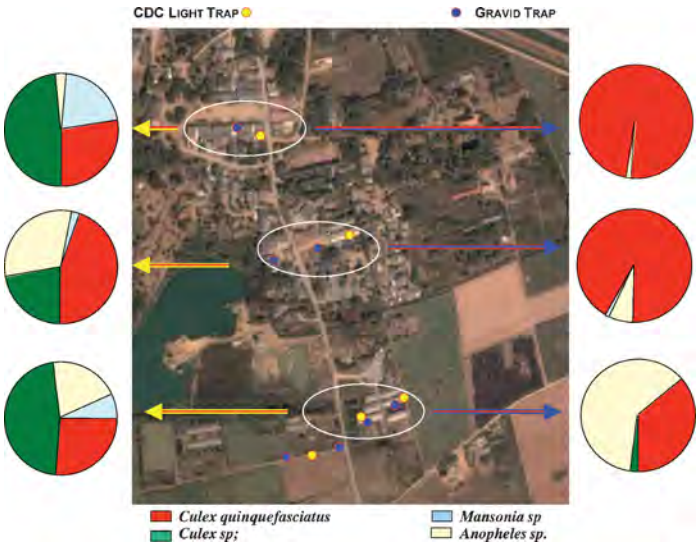


Fig. 32.7. Pig farm study site (obtained by Google° Earth). See text for full caption.

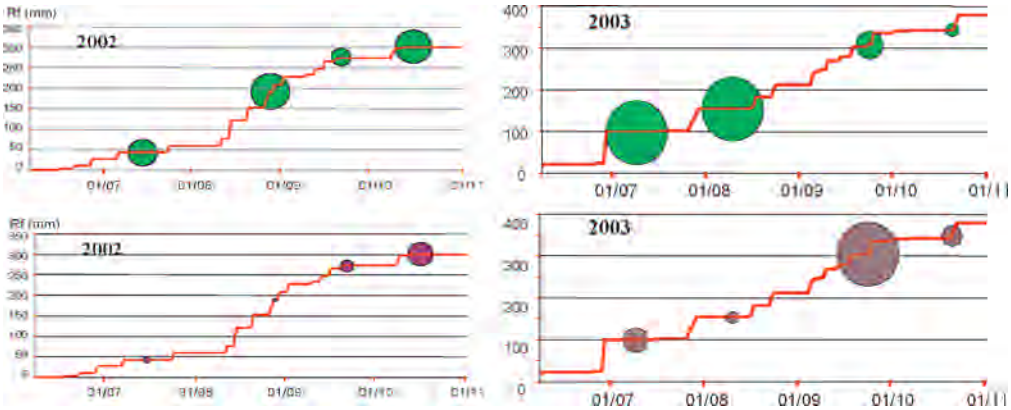


Fig. 32.10. Breeding site dynamics of Rift Valley Fever vectors: evolution of cumulative rainfall and abundance variations of *A. v. arabiensis* and *C. poicilipes* females. See text for full caption.

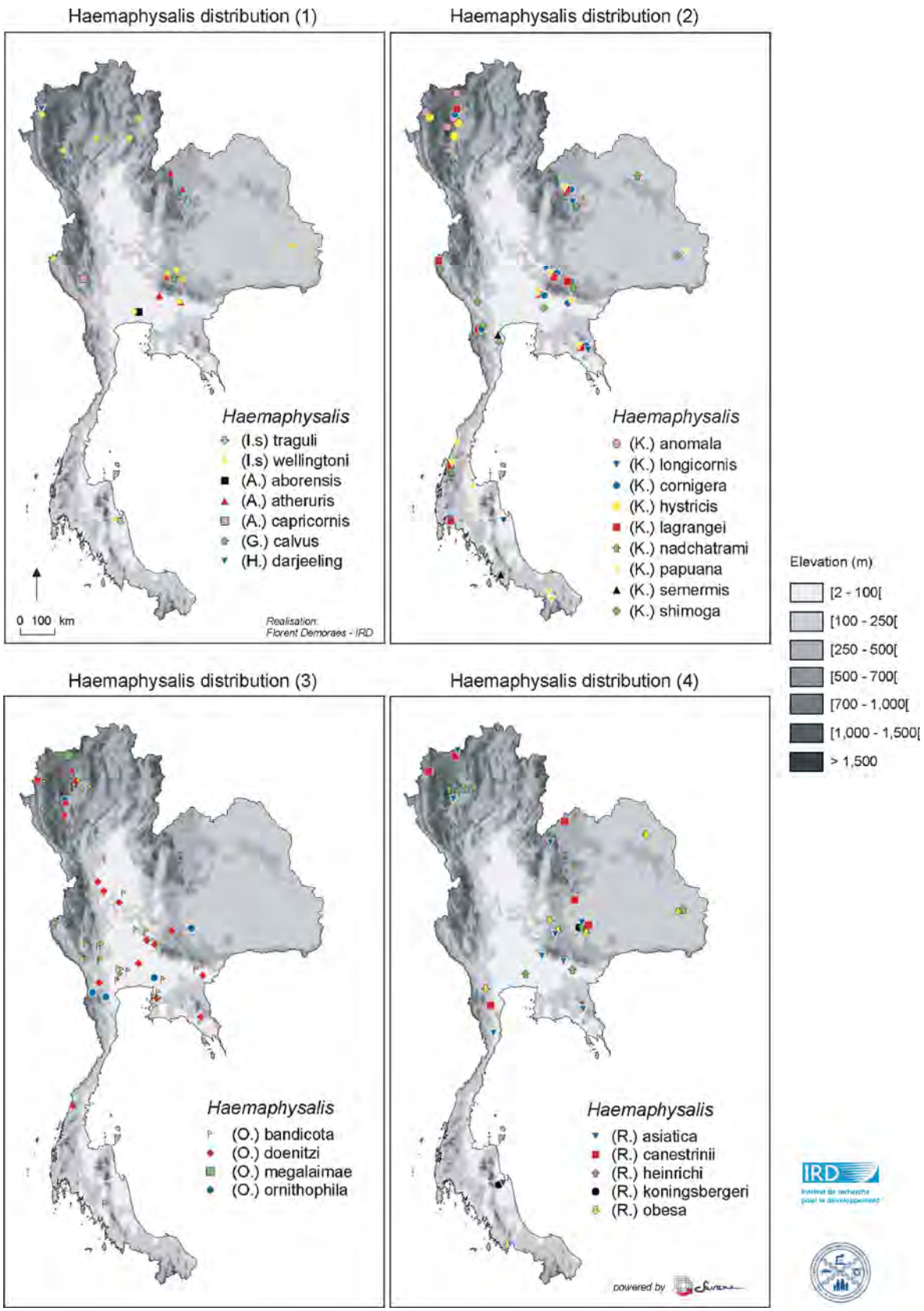


Fig. 32.13. Distribution of *Haemaphisalis* genus in Thailand.

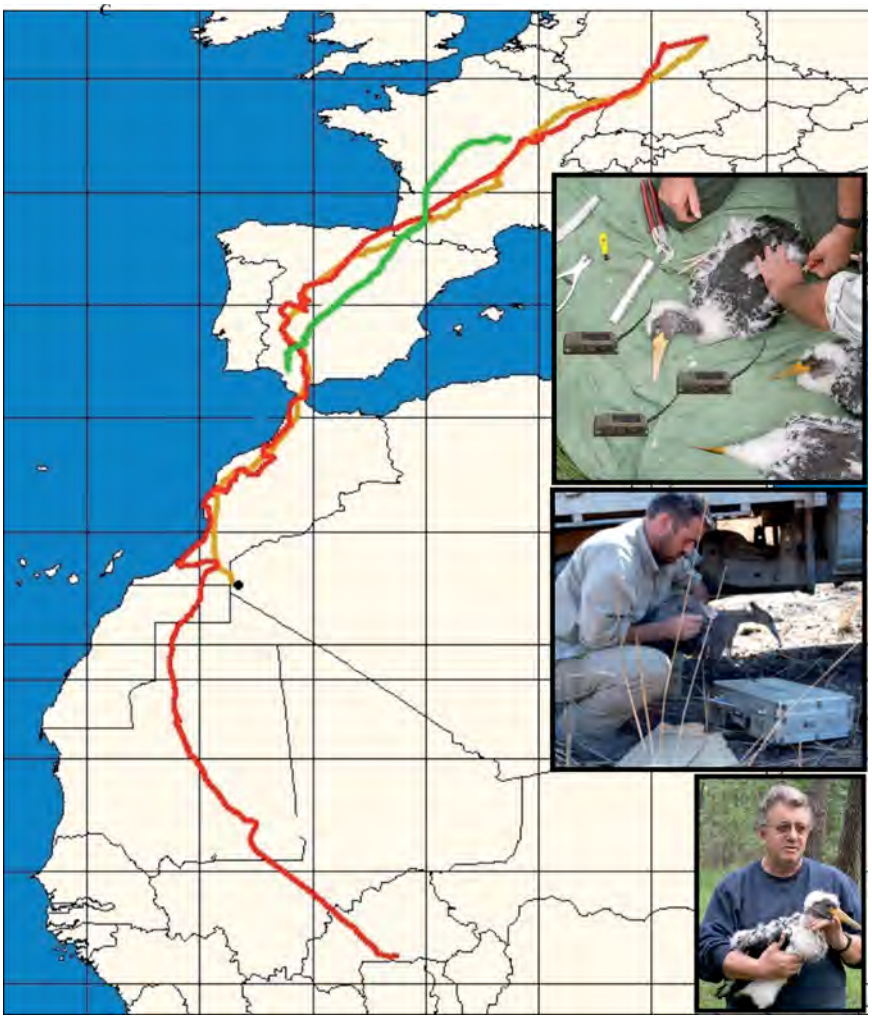


Fig. 32.16. Tracking birds for a follow up on their potential role of pathogen dispersion. *Top to bottom*: Biological sampling (blood, swabbing) an Ombrette in Burkina Faso; storks ringed with four solar transmitters Argos—GPS with their antenna visible ready to be equipped; the transmitter in place on the back of bird ready to be set free. *Background map*: Migration route of Black Stork (2004–2005); red line: Aurélia: Migration outward journey (April–June 2004); orange line: Aurélia: Migration Return (July–September 2004); green line: Camille: Migration outward journey (August–October 2005). *Left to right, from top to bottom*: (A) biological sampling (blood, swabbing) on a Hamerkop in Burkina Faso; (B) Young Storks captured to be ringed and equipped with GPS Argos solar transmitters; (C) Young Storks to be ringed, transmitters with their antenna are visible next to birds ready to be equipped. (C) Argos GPS solar transmitter placed on bird's back, ready to release bird.

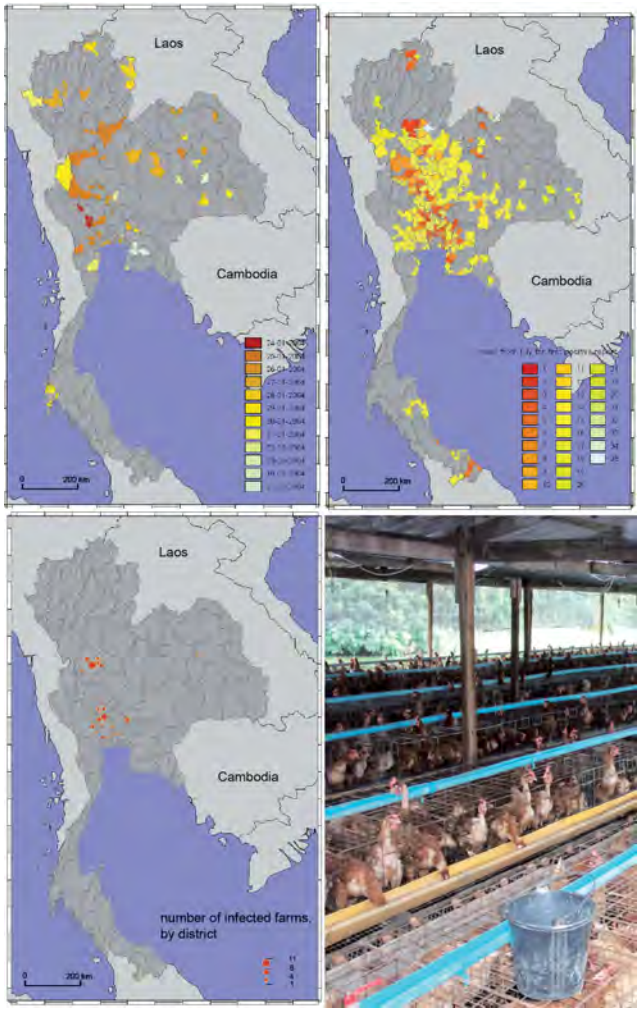


Fig. 32.18. Highly pathogenic avian influenza in Thailand. See text for full caption.

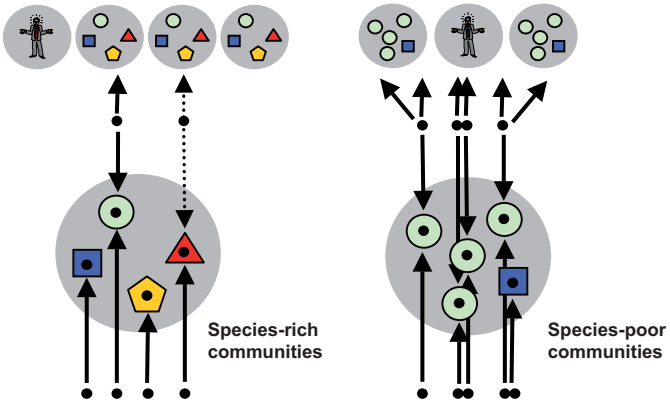


Fig. 33.7. Schematic representation of the ecology and epidemiology of transmission of Lyme vector-borne disease. See text for full caption.

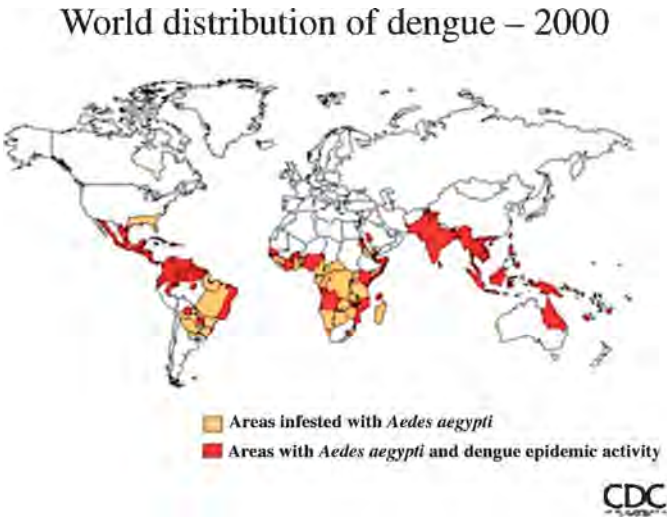


Fig. 35.5. Distribution of *Aedes aegypti*-infested areas and dengue epidemic areas (www.cdc. gov/.../dengue/ map-distribution-2000.htm).

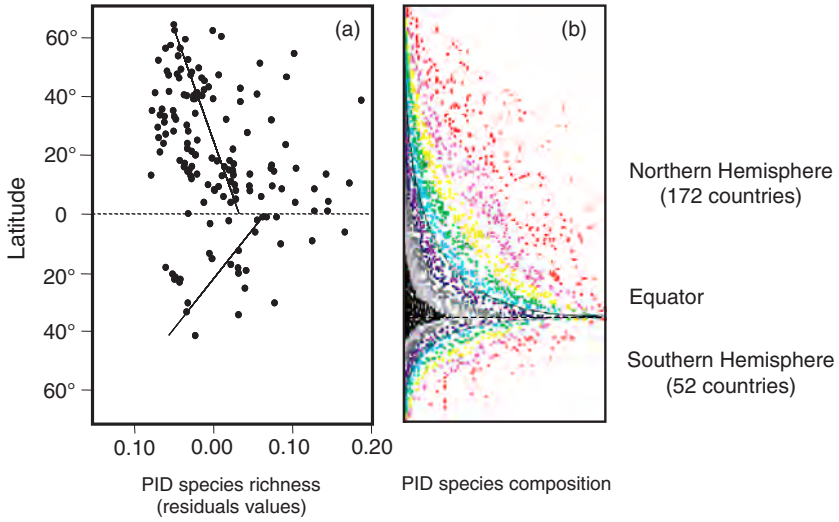


Fig. 33.8. (A) Evolution of parasitic and infectious disease (PID) species richness with latitude across the two northern and southern hemispheres. See text for full caption.

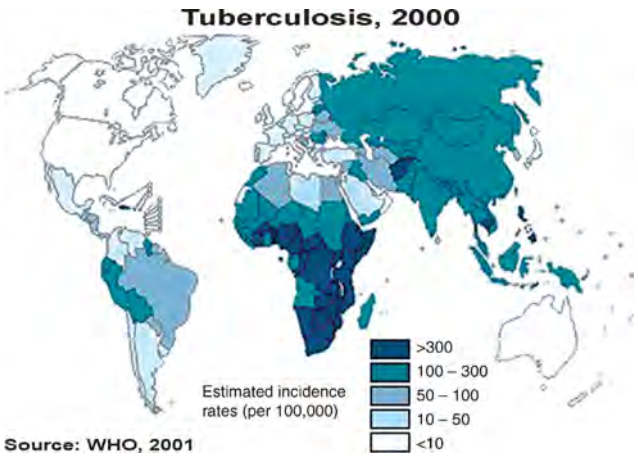


Fig. 35.6. Incidence rates for tuberculosis worldwide (www. travel-vacs.ca/. . . / Fact_Sheets12.cfm).

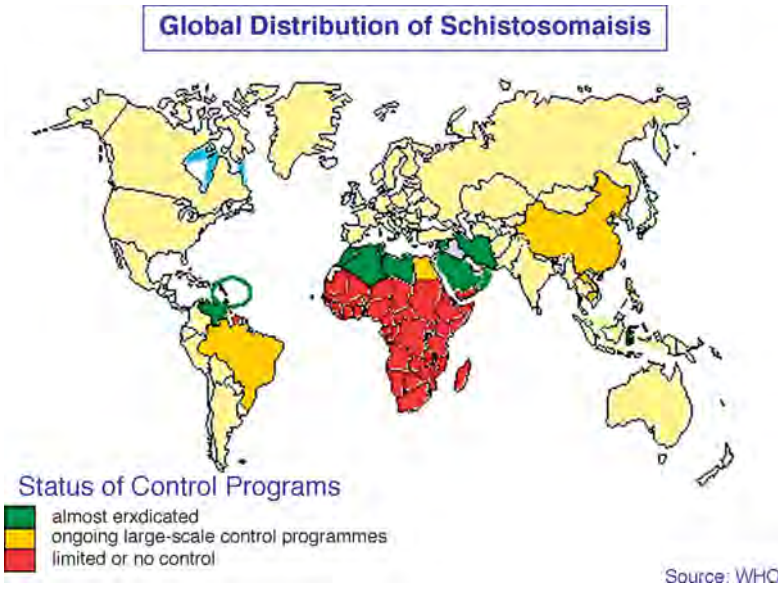


Fig. 35.7. Distribution and control programs of schistosomiases (www.schisto.org/. . . / global_ distribution. htm).

Fig. 38.4. VITEK—automated specimen-processing and handling system (BioMerieux).

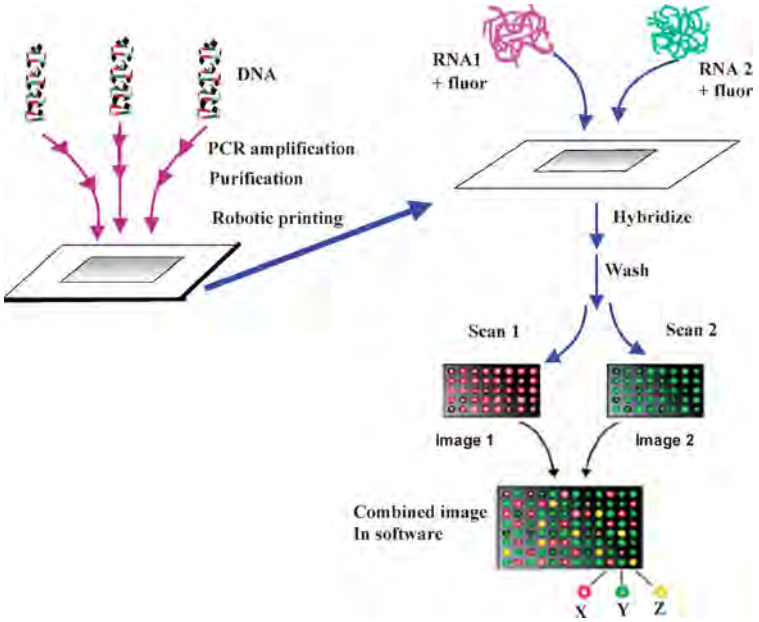


Fig. 38.5. Schematic presentation of microarray technology. See text for full caption.

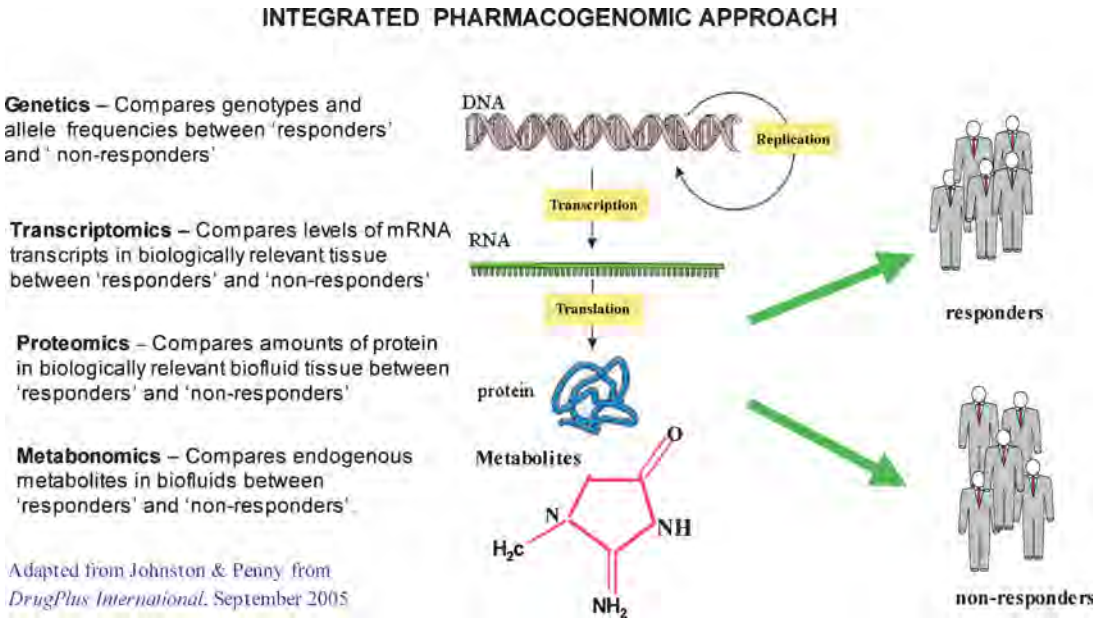


Fig. 38.7. Integrated pharmacogenomic approach to discern responder from nonresponders to drugs. (Adapted from Johnston G and Penny M, DrugPlus International 2005;4:19–22.)

Fig. 40.2. *The Triumph of Death* by Pieter Bruegel the Elder (toward 1562). Death is in the center of the painting. It is armed with a scythe and is astride an emaciated horse. It is pushing Man into a large box whose door is marked with a cross—clearly a trap door. A hoard of skeletons is invading the scene. They are striking down everyone, with no exception: the king, the mother, and her baby; the knight and the fair ladies; the peasant and the lovers playing music who do not notice the presence of a skeleton behind them. Card players are defending themselves with swords; they have not understood that all resistance is futile. The landscape reflects this death: the trees and grass are dried out, behind the hills there is an infernal fire burning, skeletons everywhere are slaughtering humans: drowning them, hanging them, cutting their throats. A man about to be decapitated is praying in vain, his rosary in his hands. Any promise of redemption or resurrection is absent in this painting. Skeletons are sounding the knell, and there is no hope.

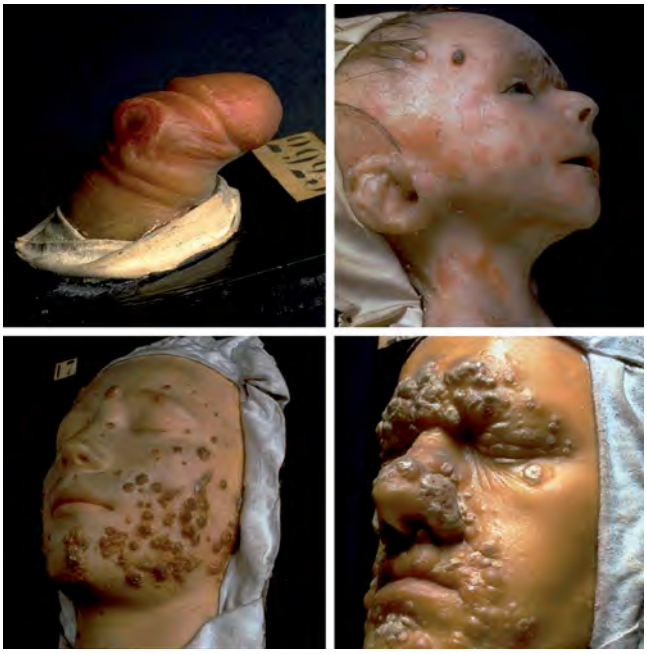


Fig. 40.6. Wax sculpture (Jules Baretta (1834–1923) Moulages 2923, 11, 17, 208 Jules Baretta (1834–1923) Collection générale de l'Hôpital Saint Louis. Extrait de: Le musée des moulages de l'hôpital Saint-Louis. G. Tilles et D. Wallach, 1996. Ed. APHP et Doin.

Fig. 40.10. *Death and Life* (G. Klimt 1916) oil on canvas, 178 × 198 cm Vienna, Dr Rudolf Leopold Collection.

Fig. 40.11. *Les Demoiselles d'Avignon*. Pablo Picasso, 1907. Museum of Modern Art, New York. Oil on canvas (224 × 234 cm).

Fig. 40.12. *Woman Putting on Her Stocking*, by H. de Toulouse-Lautrec (1864–1901) (domaine publique).

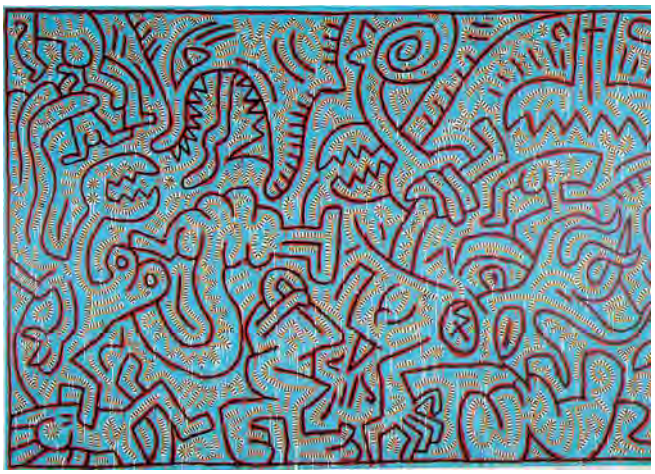


Fig. 40.14. Untitled (Keith Haring. 1984) acrylic on muslin tarpaulin 120 × 180 in private collection.

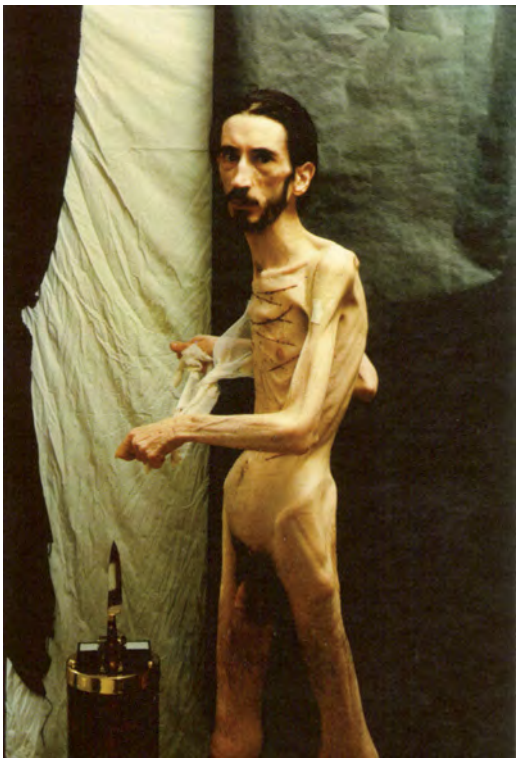


Fig. 40.18. Nude and Mother gift. David Nebrada 1989 "My mother gift: The knife with my name on it." David Nebrada. Color picture on argenetic paper, 102 × 75 cm Paris, galerie Leo Scheer.

CHAPTER 1

Pulmonary Tuberculosis and *Mycobacterium Tuberculosis*: Modern Molecular Epidemiology and Perspectives

Sylvain Godreuil,[1] Loubna Tazi,[2] and Anne-Laure Bañuls[1]

[1]*Génétique et Evolution des Maladies Infectieuses (UMR CNRS/IRD 2724), IRD de Montpellier, 911 av Agropolis BP 64501, 34394 Montpellier Cedex 5, France*

[2]*Department of Microbiology & Molecular Biology, Brigham Young University, 775 Widtsoe Building, Provo, UT 84602, USA*

1.1 INTRODUCTION

Tuberculosis (TB) is a bacterial infection caused mainly by *Mycobacterium tuberculosis* (MTB). The development of paleopathology and paleoepidemiology in infectious diseases has proven the *very ancient origin* of this disease. TB may have plagued humans at least since the Neolithic times [64,158,186]. This infectious disease was sporadic until the 1700s and became epidemic afterward because of the *industrial revolution*, the *increase in population density*, and *unfavorable living conditions*. Furthermore, human migrations and colonization of countries and continents helped to spread TB, which became an endemic disease. In 1882, *Robert Koch* managed to isolate the tubercle bacillus (called also *Koch bacillus*), the bacterium responsible for TB, and he established TB as an infectious disease. Over the last 100 years, TB has probably killed 100 million people [66]. In the twentieth century, the incidence of this disease began to decline rapidly in developed countries where the sanitation and housing conditions were improved. This scenario was accelerated by the introduction of *BCG vaccine* (Bacillus Calmette Guérin, 1921) and the use of antimicrobials as anti-TB agents, such as *streptomycin* (1943), *isoniazid* (1952), and *rifampin* (1963). However, despite these efforts to eradicate this disease, the incidence of TB increased in the 1980s. The emergence of *multidrug-resistant strains* and the *high incidence of human immunodeficiency virus* (*HIV*) have strongly contributed to this phenomenon. Nevertheless, the success of propagation of this disease remains directly related to the *social and hygiene conditions* of human populations.

TB remains a major public health problem worldwide and the first cause of mortality attributable to a single infectious agent, especially in developing countries where the consequences of this disease remain more serious and the infection risks are higher [54,148] (see Fig. 1.1). According to the World Health Organization (WHO, www.who.int), the estimated number of cases of TB worldwide in 2003 was 8.8 million, 3.9 million of which were sputum positive, and deaths from TB (including TB deaths in people infected with HIV) were 1,747,000. Furthermore, it is estimated that there are currently 2.1 billion people worldwide who are latently infected with the tubercle bacillus and could develop the active form of the disease in the case of reactivation.

The recrudescence of this disease in several countries, the emergence of multidrug-resistant strains and the association of TB with the HIV pandemic show the need to improve research on this pathogen in applied and basic research in order to better understand the transmission of TB and to eradicate this disease. The objective of this chapter is to give an overview of the biology, genetics, and pathogeny of *M. tuberculosis* (MTB), to describe the current molecular methodologies available for identifying the MTB populations responsible for the spread of TB and the outbreaks, and to show the contribution of genetic epidemiology studies in understanding global and local epidemiology of TB.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

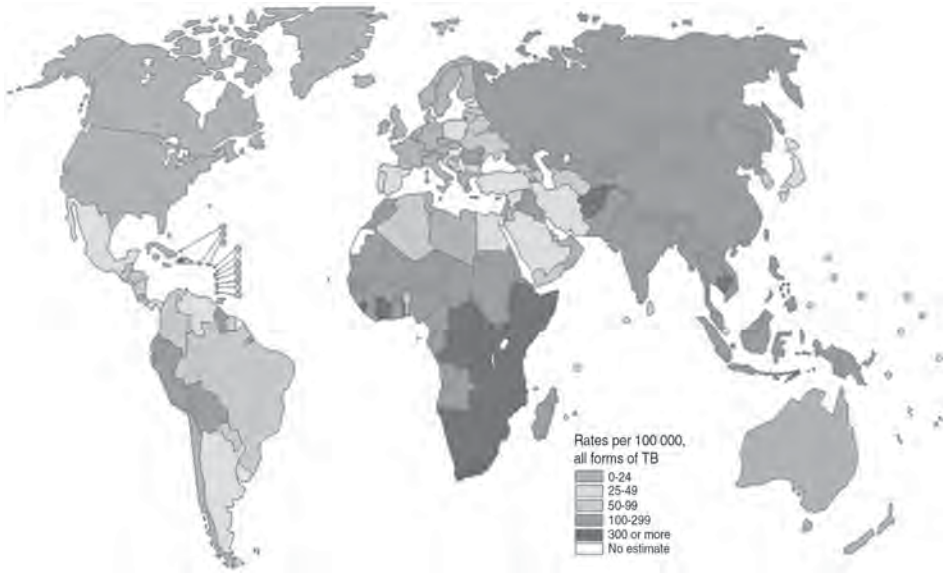


Fig. 1.1. Estimated TB incidence rates, 2003 (WHO website: http://www.who.int/tb/publications/global_report/2005/results/en/index.html)

1.2 GENERAL POINTS ON *MYCOBACTERIUM TUBERCULOSIS* (MTB) AND PULMONARY TUBERCULOSIS (PTB)

1.2.1 Classification and Cellular Characteristics

MTB is a bacterium belonging to the *Mycobacterium* genus, which is the only genus in the Mycobacteriaceae family (Chester, 1897), Actinomycetales order (Buchanan, 1917), and Actinomycetes class (Krasil'nikov, 1949).

The *Mycobacterium* genus, one of the most extensively studied bacterial taxa, was described by Lehman and Neuman in 1896. Its identification is based on the following characteristics: shape of the colonies, growth rate, and biochemical reactivity. To date, 71 species have been described within this genus, and they are subdivided in two main groups based on their growth rates (fast vs. slow) [109,154,163]. The rapidly growing *Mycobacterium* species (species producing visible colonies within 7 days under optimal culture conditions) are mainly common saprotrophs of natural habitats. Only a few of them can be pathogenic for humans or animals (e.g., *Mycobacterium abscessus*, *M. fortuitum*, *M. porcinum*), whereas the majority are nonpathogenic (e.g., *M. smegmatis*, *M. agri*). In contrast, the majority of the slowly growing *Mycobacterium* species are pathogenic for humans and/or animals (e.g., all the species of the MTB complex [MTBC], *M. leprae*, *M. ulcerans*, *M. avium*), and only a few of them are nonpathogenic (e.g., *M. terrae*, *M. gordonae*). This chapter will focus particularly on the *MTB species* belonging to the MTBC. This complex is composed of seven different species, MTB (Koch, 1882), *M. bovis* (Karlsen and Lessel, 1970), *M. africanum* [25], *M. microti* (Reed, 1957), "*M. canettii*" (still not officially recognized on the list of Bacterial Names with Standing in Nomenclature, http://www.bacterio. cict.fr), and recently, *M. caprae* [5] and *M. pinnipedii* [34]. Each member of MTBC is associated with a specific primary host, although infection is known to occur in various alternative hosts. The species responsible for TB in humans and for which no animal reservoirs was found are MTB, *M. africanum*, and *M. canettii*. MTB is the main species of human TB, the other species are less frequent in humans (e.g., *M. africanum* is characterized mainly in Africa, and *M. canettii* was isolated in a few cases of human TB in East Africa). *M. bovis* is principally the agent of bovine TB, but this species can also be pathogenic for humans, with the number of cases related to such infection probably underestimated [6]. Furthermore, an attenuated strain of *M. bovis*, *M. bovis BCG* (Calmette and Guérin, 1921) is used as a *vaccine* for preventing human TB (see below for more details on BCG vaccine). The other species are also isolated specifically in animals, such as *M. microti*, which is the agent of rodent TB, *M. caprae*, which predominantly affects cattle, and *M. pinnipedii*, which has Pinnipeds as natural hosts. These latter species can affect other animal species and humans to a very limited extent [5,34]. The members of the MTBC, as well as all mycobacteria species, are *rod-shaped bacteria* (0.2–0.6 μm wide, 1–10 μm long), nonmotile, nonencapsulated, Gram-positive, aerobes (growing most successfully in tissues with a high oxygen content such as lungs), or facultative anaerobes. They are *facultative intracellular pathogens*, usually infecting mononuclear phagocytes (e.g., macrophages). As deduced from its genome, MTB has the potential to manufacture all of the machinery necessary to synthesize its essential vitamins, amino acids, and enzyme cofactors. *MTB* has an unusual cell wall, with an additional layer beyond the peptidoglycan layer, which is rich in unusual lipids, glycolipids, and polysaccharides. These bacteria can be detected by optical

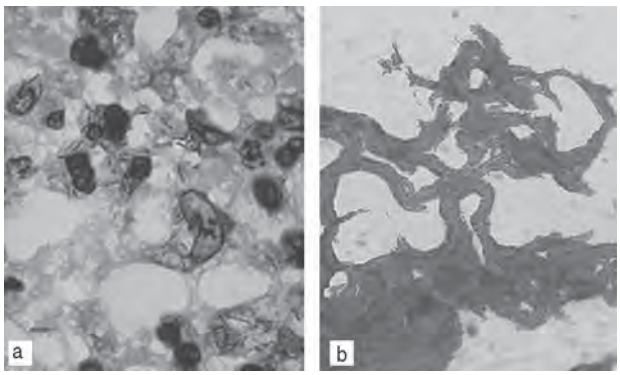


Fig. 1.2. *Mycobacterium tuberculosis* (MTB): *Ziehl–Neelsen* coloration (a) from sputum; (b) from MTB culture in liquid medium (photo taken by S. Godreuil, all rights reserved). See color plates.

microscopy after *Ziehl–Neelsen* (*ZN*) *acid-fast stain* of sputum from a person with active TB (Fig. 1.2). Bacilli appear as thin red rods in the microscopic field, whereas all other materials in the sputum pick up the blue counterstain.

1.2.2 Transmission and Multiplication of MTB (See Also Chapter 5)

TB is considered a disease with an *interhuman transmission*. Tuberculous bacilli are spread out by infected patients coughing, sneezing, or speaking, and they can be inhaled by another individual in close contact. The inhalation of these sprays, called Flugge's droplets—small aerodynamic particles—presents a risk of tuberculous infection. These particles can also remain in the air and play the role of reservoir.

The tubercle bacillus enters the human body mainly via the respiratory tract through the inhalation of the droplets sprayed in the air (Fig. 1.3). These particles are small enough to be able to reach the lower respiratory tract. Indeed, among the infectious particles inhaled, only those with two or three bacilli can reach the bronchic cells, the largest ones are stopped upstream and eliminated [44]. The success of such infection and the development of the pulmonary form of TB depend on four successive stages: bacilli phagocytosis, intracellular multiplication, the stationary stage, and the pulmonary form of TB (see also Chapter 5). These different stages can evolve into different outcomes: spontaneous healing, acute tuberculosis, latent infection, and reactivation or reinfection (see Fig. 1.3).

(i) *Bacilli phagocytosis*: The bacilli that reach the pulmonary alveolus are phagocyted by the mature macrophages. This step, which takes place in the first week following particle inhalation, is the first stage of infection, and it depends on two main factors: the bacillus virulence and the bactericidic activity of the macrophage. In general, the bacteria are destroyed by the alveolar macrophages and the infection is stopped at this stage, otherwise they begin an intracellular cycle of multiplication [119,179].

(ii) *Intracellular multiplication*: This second stage occurs between the 7th and the 21st day. It corresponds to intracellular bacilli multiplication in the macrophage alveoli and is also called the symbiotic stage. Indeed, the bacteria that are not destroyed by the alveolar macrophages will multiply. They are released after cellular lysis, and can thus infect other circulating macrophages and continue their multiplication. At the end of this stage, due to a symbiosis event, a huge number of macrophages and bacilli are concentrated at the level of early pulmonary lesions [44].

(iii) *Stationary stage*: Following the induction of the immune response of the host, particularly cell-mediated immunity

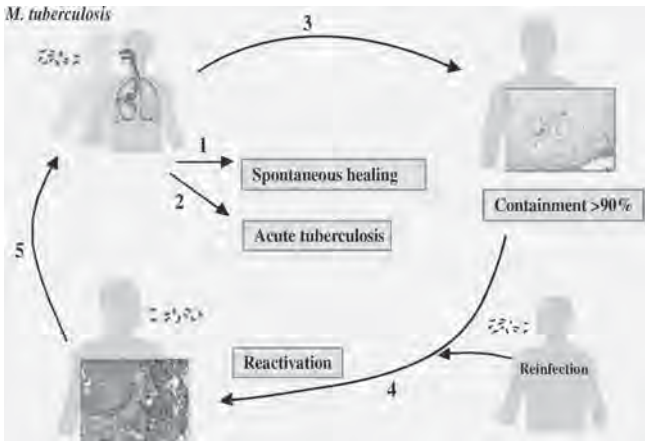


Fig. 1.3. Inspired by Kaufmann and McMichael [96] with permission: *Mycobacterium tuberculosis* (MTB) enters the host within inhaled droplets. Different outcomes are possible. (1) Immediate eradication of MTB by the pulmonary immune system. (2) Infection transforms into active tuberculosis. (3) Infection does not transform into disease because MTB is contained inside granulomas. (4) After a latency phase, MTB can become active after either an endogenous reactivation or an exogenous reinfection or both. (5) At this stage, there is dissemination and transmission of MTB.

(see Chapter 5), bacteria growth becomes stationary [142]. This is the third stage of the infection called *primary infection*. Because of a delayed-type hypersensitivity, the macrophages in which bacilli multiply are destroyed. Bacterial toxins and cellular products are released, and this leads to the formation of solid caseous necrosis [137], where a pseudo-equilibrium settles between inactivated and mature macrophages. At this stage, either the number of infected cells in the caseous center decreases if the released bacilli are phagocyted by the mature macrophages or it increases if the bacilli multiply in the inactivated macrophages. Thus, the progression of the disease depends on which macrophage type prevails [43,44]. At this stage, bacilli may become dormant and never induce TB at all, which is referred to as a *latent infection* that is detected only by a *positive tuberculin skin test*; or the latent organisms can eventually begin to grow, with resultant clinical disease, known as TB reactivation.

(iv) *Pulmonary form of TB* (*PTB*): When the equilibrium between the inactivated and mature macrophages is broken, the infection reaches the last stage, the disease, PTB (see also Chapter 5). This step is characterized by the liquefaction of the caseous center, leading to the formation of a cavity detected by pulmonary radiography. The liquefied material present in this cavity constitutes an excellent growth media for the bacteria, and macrophages do not survive in this environment. *At this stage of the disease, the person becomes contagious by releasing the bacilli into the air.* Furthermore, without treatment, this individual can develop a chronic TB, presumably leading to death.

1.2.3 Clinical and Subclinical TB

The term "TB infection" refers to a positive TB skin test (see below for details) with no evidence of active disease; this state is also called latent infection (see Fig. 1.3). "*TB disease*" *refers to cases that have a positive acid-fast smear or culture for MTB or radiographic and clinical presentation of TB* [117].

The most common clinical manifestation of TB is pulmonary disease; nevertheless, extrapulmonary TB can also occur, but is little or not contagious. Without minimizing the importance of extrapulmonary TB, which currently accounts for 20% of reported cases of TB, we will focus here only on the cases of pulmonary infections. Furthermore, as described above, although some people develop active TB disease after infection, *almost all TB infections are asymptomatic and remain latent* [19].

1.2.3.1 Active disease A patient with PTB presents with the symptoms of chronic or persistent cough and sputum production. If the disease is at an advanced stage, the sputum will contain blood, and the patient will be diagnosed with lack of appetite, weight loss, fever, night sweats, and thoracic pains. Patients with PTB are classified in different categories because a specific treatment is needed for each category. The main categories are as follows:

- New case: TB in a patient who either has never received anti-tuberculous treatment or started a treatment for less than 1 month.
- Relapse: TB already treated and declared cured after sufficient treatment time, which has become active again.
- Chronic TB: A case of relapse from which the microscopic exam of expectoration remains positive after a second complete treatment.
- Primary resistance case: This characterizes the bacilli that are resistant to treatments, although patients have never been treated by anti-tuberculous drugs (see below).
- Multiresistance case: MTB resistant at least to both major anti-tuberculous drugs (isoniazid and rifampin) (see below).

1.2.3.2 Latent infection MTB in a latent state can subsequently reactivate to cause active disease. The latent state of infection is a major obstacle for eradicating TB. In latent TB, the host immune response is capable of controlling the infection but fails to eradicate the pathogen. Latent TB is the product of a complex set of interactions between MTB and the host immune response (for more details, see Chapter 5). Therefore, one-third of the world population is estimated to be infected with the pathogen in the latent stage. The bacilli remain dormant until the host defenses are impaired by a disorder such as HIV infection.

1.2.3.3 MTB and HIV *For many people, TB is the first sign of immune dysfunction associated with HIV infection, and active TB is an AIDS-defining illness*. TB is an ever-increasing concern for people with HIV. In some parts of the world, TB is the leading cause of death of people infected with HIV. Indeed, the risk of developing active TB disease after TB infection, or following an apparent cure of several years, increases considerably for people with a deficient immune system. It was calculated that in case of HIV co-infection, this risk is multiplied 50–300 times [101]. Active TB in HIV-positive patients can result from both reactivation of latent infection and primary disease. HIV increases the chance of reactivating dormant TB infection from 5% to 10% over a person's lifetime to 7% to 10% per year. In patients with low CD4 cell counts, TB arises with atypical pulmonary manifestations and extrapulmonary disease. Indeed, as the level of immunodeficiency increases with advancing HIV disease, atypical pulmonary features predominate [31]. One in 10 people living with HIV will get active TB within 1 year of being diagnosed with HIV. It can occur early in HIV disease when CD4 cell counts are relatively high, in the 300–400 range. In early HIV infection, TB usually infects and affects only the lungs. As CD4 cell counts drop, however, TB is more likely to appear in other organs also. When the immune system responds to TB, it can cause HIV levels to increase, and HIV disease may then progress more quickly. This, in turn, increases the risk of other opportunistic infections. *It is therefore very important for people with HIV to be screened regularly for TB.*

1.2.4 Diagnosis of MTB Species

1.2.4.1 Tests for active disease Tools for the diagnosis of active disease include clinical suspicion, response to treatment, chest radiographs, staining for acid fast bacilli, culture for mycobacteria, and, more recently, nucleic acid amplification assays (for more details, see review in [19]). Briefly, as described above, TB can mimic many forms of disease and must always be considered if no firm diagnosis has been made. The *chest X-ray examination* is traditionally considered as one of the most important tests, but its low specificity can lead to overdiagnosis. To confirm the diagnosis of PTB, respiratory samples (expectorated sputum) are submitted to the bacteriological laboratory for *microscopic examination* and for *mycobacterial culture*. The microscopic examination consists of making a smear of sputum and staining by the *Ziehl–Neelsen* (*ZN*) *method* (see Fig. 1.4). This technique is used in most low-income countries because it is inexpensive and easy to use [144], but its low sensitivity (43–55%) is a major drawback [214]. Cultures increase the sensitivity for diagnosing MTB and allow drug-susceptibility testing and genotyping for epidemiological purposes (see below). Nevertheless, culturing TB is time consuming and the cost is often too high, resulting in reliance solely on microscopy of sputum smear in resource-poor countries [19]. Two types of culture media are commercialized: solid media, which includes egg-based media (Lowenstein-Jensen; see Fig. 1.4), and liquid media (such as BACTEC systems, Becton Dickinson, Sparks, MD, USA). Several studies showed that liquid media can decrease recovery time (2 weeks instead 4–12 weeks) of mycobacteria culture and increases the sensitivity compared to solid media, which remains the reference media for culturing mycobacteria [124,162]. The traditional methods of drug-susceptibility testing relied on culture inoculated with antibiotics and thus can also require several weeks to obtain results.

These methods remain the gold standard for diagnosis, but the development of DNA probes and the polymerase chain reaction (PCR) assays now provide more sensitive and rapid diagnosis for species identification as well as for analyzing drug susceptibility. Currently, two main methods approved by the Food and Drug Administration (FDA) are available: a PCR-based test targeting a specific portion of the 16S ribosomal RNA gene (Roche) and a transcription-mediated amplification of 16S ribosomal gene transcripts with product detection performed via chemiluminescence (GenProbe). Furthermore, Kaul [97] as well as Brodie and Schluger [19] detailed in their reviews all the latest techniques based on nucleic acid amplification. These diagnostic tests have considerably decreased the diagnosis recovery time and increased the sensitivity for smear-positive and smear-negative specimens. These techniques are the most promising methodologies for diagnosing the 15–20% of adults with TB having negative sputum culture and among children, for whom the proportion of culture-negative cases is much higher. At present, the greatest problem concerning these techniques is the cost; consequently, they are not affordable for resource-poor countries [97].

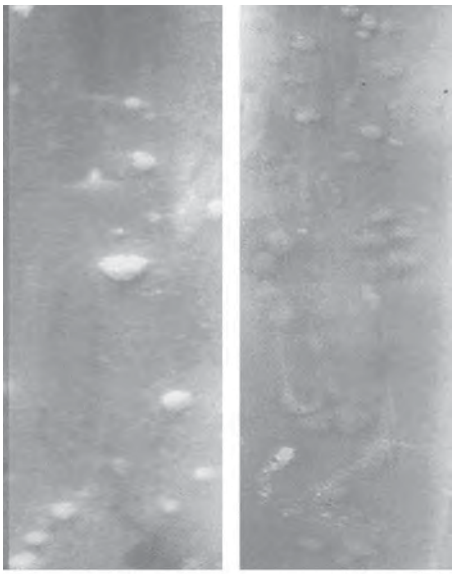


Fig. 1.4. Colonies of *Mycobacterium* on Lowenstein–Jensen. (a) *M. tuberculosis* colonies are irregular, rough, eugonic, beige in color. (b) Atypical mycobacteria colonies are small, smooth, and pigmented (photo taken by S. Godreuil, all rights reserved). See color plates.

During the past few years, there has also been great progress in exploring drug susceptibility in MTB. In their review, Brodie and Schluger [19] described the latest methodologies allowing for a rapid detection of the drug-resistant mutations from smear-positive respiratory specimens or from culture specimens with their limits and advantages: line probe assays, molecular beacons, phage amplification, and luciferase reporter phages.

1.2.4.2 Tests for latent infection It has been demonstrated that almost all TB infections are asymptomatic and remain latent, with a rate of *reactivation* in active disease in approximately 5–10% of infected individuals. These people are a reservoir for the disease and a major barrier to the ultimate control and elimination of TB. Until very recently, a skin reaction Mantoux test or tuberculin skin test or PPD (purified protein derivative) skin test was the only available test to detect latent disease or to confirm the cases of active disease with negative sputum smear or culture. Nevertheless, this test presents various problems such as relatively poor sensitivity and specificity. Recently, a new generation of tests has been developed: QuantiFERON-TB and QuantiFERON-TB Gold (QFN-Gold) tests (Cellestis Limited, St. Kilda, Australia) and the T SPOT-TB test (Oxford Immunotec, Oxford, UK). These tests are based on the detection in serum of either the release of IFN-γ (QuantiFERON) or detection of the T cells themselves (T SPOT-TB) (for more details see review of Brodie and Schluger [19]). These tests seem to improve specificity and sensitivity. At present, in most high-burden, resource-poor countries, latent infections are neither diagnosed nor treated. However, for the TB control and for

stopping the disease progression, it would be imperative to treat the latent infections.

1.2.4.3 Diagnosis in smear-negative PTB and the special case of HIV patients Patients with smear-negative PTB have been found to be less infectious and have a lower mortality rate, but a significant proportion (50–71%) progressed to active disease warranting treatment [31].

Furthermore, several studies showed that with the increase in the HIV/TB coinfection, there has been a disproportionate increase in the reported rate of smear-negative disease (see review in [31] for details). Indeed, different data reports suggest that smear-negative disease is actually more common among HIV-infected patients. Colebunders and Bastian [31] hypothesize that smear examinations have proven less sensitive, as the level of immunodeficiency has been increasing with advanced HIV disease. This is a crucial problem in developing countries where microscopic examination (cheap, simple, and rapid) is the basis of TB diagnosis. Nevertheless, several studies showed that also in HIV-uninfected populations, a non-negligible rate of smear-negative PTB can be observed (see [31] for details). This is normally associated with low bacilli burdens and minimal pulmonary lesions. Furthermore, this is especially more common among children and elderly patients.

As described above, in HIV patients, atypical pulmonary features predominate and chest radiography changes may be atypical or attributable to other infections. Furthermore, the tuberculin skin testing is confounded, especially in developing countries presenting a high rate of HIV/MTB co-infection, with the high coverage of BCG vaccination, with asymptomatic TB infection, with the presence of nontuberculous mycobacteria, and with anergy due to HIV or malnutrition.

Culture and PCR remain the most sensitive techniques, as they can produce a positive result for specimens containing as few as 10 bacilli. This is of a great interest, as HIV-positive patients generally produce sputum with low bacilli loads [31]. Nevertheless, these diagnostics are still financially inaccessible for resource-poor countries.

For these reasons, some authors have proposed various management algorithms to optimize the number of patients correctly treated for smear-negative sputum and thus a major part of HIV-positive patients. In their review, Colebunders and Bastian [31] detail the different parameters included in these management algorithms. They are based on the following combined features: clinical symptoms, response to antibiotic trials, smear investigations, and chest radiography.

1.2.5 Treatment, Drug Resistance, and Control

1.2.5.1 Treatment According to the current recommendations, *effective TB drug therapy requires at least two effective drugs* [157]. Sahbazian and Weis [157] detail how this axiom has emerged to limit drug resistance, which is, along with lack of patient observance, the most important factor of chemotherapy failure. These authors also review available drugs and their toxicity [157]. Of the drugs approved by the Food and Drug Administration (FDA), isoniazid, rifampin, ethambutol, and pyrazinamide are considered first-line anti-TB drugs. Rifapentine and rifabutin can also be considered as first-line drugs under special conditions. The others (see review of Sahbazian and Weis [157]) are categorized as second-line drugs, which are used when the first-line drugs are unsuitable because of drug intolerance or infection with drug-resistant TB. The WHO's Stop TB Department, with the help of the International Union Against TB and Lung Disease and experts worldwide, has published guidelines for a standardized and efficient treatment of TB (http://www.who.int/tb/en/index.html). They give practical guidance for national TB programs and for the medical profession in the effective management of TB. Different targets of TB treatment are reviewed, such as principles of treatment, with an update of the guidelines, care in the context of HIV/TB infection, multidrug-resistant TB (MDR-TB), and chronic diseases. Guidelines for high-income and low-incidence countries, even though they follow the same principles, include recommendations that may not be appropriate for most high-incidence countries where resources for TB control are often limited. The most cost-effective public health measure for the control of TB is the identification and cure of infectious TB cases, that is, patients with smear-positive PTB. Nevertheless, national TB programs provide guidelines for identification and cure of all patients with TB. These guidelines cover the treatment of patients, both adults and children, with smear-positive PTB, smear-negative PTB, and extrapulmonary tuberculosis. *It is important to note that TB treatments require long-term drug administration, which is logistically difficult and generally results in uncontrolled disease burden in developing countries*.

1.2.5.2 Vaccination *Vaccines are desperately needed* because of several factors such as duration and cost of existing treatment, cost of diagnosis, rate of drug resistance, difficult access to cure for poor-resource populations, and the high rate of latent infections. At present, the only available vaccine is the *BCG* (*M. bovis* bacillus Calmette–Guérin), which is a deletional mutant of *M. bovis* that arose spontaneously during subculture on beef-bile-potato medium [135]. In their review, Rook et al. [155] expose the limits of BCG and demonstrate that while it helps to protect against childhood forms of TB, it provides variable protection in adults and it has a minimal impact on disease control in developing countries where the vaccine is most needed. According to Hampton [84] and Ginsberg [73], hundreds of new TB vaccine candidates are under study, including subunit vaccines, consisting of immunogenic mycobacterial components; DNA vaccines; live, attenuated mycobacteria; and live, attenuated nonmycobacterial vectors, such as *Salmonella* or vaccinia virus [73]. Rook et al. [155] highlight that with the aim of developing a successful vaccine, it is crucial to refer to the immunopathogenesis of TB and to consider the immune response, which can differ depending on target populations

and the individual immune status (other infections, nutritional status, etc.).

1.2.5.3 DOTS strategy WHO and the International Union Against TB and Lung Disease (IUATLD) have adopted *directly observed therapy short course* (*DOTS*) as the main strategy for TB control. DOTS consists of political and administrative commitment; case detection by sputum microscopy; standardized short-course chemotherapy given under direct observation by a health professional; adequate supply of good-quality drugs; systematic monitoring; and accountability for every patient diagnosed. Frieden and Munsiff [65] review the principle, scientific basis, and experience with implementation of DOTS. According to a WHO report [213], the number of countries having adopted and launched the DOTS strategy has increased considerably since 1995, and in 2003, it had been implemented in 182 of 211 countries, covering 77% of the world's population. In 132 countries, including most of the industrialized world, DOTS is available to more than 90% of their populations. DOTS programs concur to decrease mortality rates, which are often drastically lower than in non-DOTS programs. This is truly one of the great public health success stories of the past decade. According to WHO data and details given by Frieden and Munsiff [65], DOTS has saved more than 1 million lives in the last 10 years and could save millions of lives over the next 10 years. Nevertheless, there are a number of obstacles to DOTS expansion, four of which were identified to be of overriding importance by WHO: shortages of trained staff, lack of political commitment, weak laboratory services, and inadequate management of MDR-TB and TB in people infected with HIV.

1.3 GENETICS OF MTB, MOLECULAR TOOLS, AND POPULATION STRUCTURE

1.3.1 Genome and Genetic Diversity of MTB

The genome of MTB is *haploid*, as are all bacteria genomes, and is composed of 4,411,529 base pairs (bp). It contains approximately 4000 genes and presents a rich composition in GC content (65%) [30]. This genome is characterized by the presence of numerous repeated sequences. No plasmid was detected in this species. In 1997, Sreevatsan et al. [177] studied 26 structural genes or loci, and they observed *very low levels of genetic variation*. From these results, they concluded that the genetic diversity of the species is localized, especially in transposable elements and in genes involved in host–pathogen interactions, particularly those related to host immunological responses. This last point was refuted by Musser et al. [130] after their study conducted on 24 genes coding for targets of the host immune systems. Of the 24 genes, 19 were monomorphic and the last five appeared slightly polymorphic (only six polymorphic nucleotide sites on all five genes). On the contrary, *the transposable elements show high levels of genetic polymorphism*, and they are widely used for studying the genetic variability in the MTB species [17,95,173] (see below for more details). Nevertheless, the only way to detect the real genetic diversity of an organism is the whole sequencing of several genomes from different clinical isolates. For MTB, the complete genome sequences of three strains, but also of one *M. bovis* strain, are now available (www.tigr.org) [63,70]. In addition, a sequencing project for *M. bovis* BCG is ongoing (http://www.sanger.ac.uk/Projects/Microbes). The comparison of the complete sequences of the two strains (H37Rv, which is the classical reference strain, and one recent MTB strain CDC1551) confirms a much higher degree of polymorphism than previously thought [63,82]. These latter studies made it possible to identify large-sequence polymorphisms (LSPs) and single-nucleotide polymorphisms (SNPs), whereas the molecular basis of variability in virulence and transmissibility remains undefined. Tsolaki et al. [199] have developed a complementary approach to comparative genomics involving the analysis of unsequenced genomes by DNA microarray. Although this approach is limited in the identification of relatively LSPs, it allows the comparison of a large number of genomes and thus provides information on the diversity and frequency of polymorphisms among different strains from a single population. These authors postulate that because rates of SNPs are low in this species, large sequence differences that are detectable by microarray are likely to be an important source of genetic variation. They identified 68 different LSPs (representing 186,137 bp, or 4.2% of the entire genome) that are present in H37Rv but absent from several clinical isolates. A total of 224 genes (5.5%), including genes in all major functional categories, were found to be partially or completely deleted. Deletions are not distributed randomly throughout the genome but instead tend to be aggregated. They observed that the identified deletions were evidently unessential to the development of the disease, as they were found in active clinical cases. In contrast, their frequency spectrum suggested that most polymorphisms are weakly deleterious to the pathogen. These results raise numerous opportunities to advance in the study of drug resistance, virulence, and host–pathogen interactions.

1.3.2 Genetic Tools for Molecular Epidemiology

Because it is still not possible to sequence the whole genome of MTB populations to conduct molecular epidemiology studies, in the last decade, a large number of different molecular methods based on DNA fingerprints have been developed. Several molecular techniques are available to explore the genetic diversity of MTB populations and are useful for epidemiological surveillance and understanding of TB transmission. We will detail here only the three main techniques classically used in molecular epidemiology studies. All three of these molecular tools are based on the study of transposable and repetitive elements of the MTB genome: IS*6110* (Insertion Sequence *6110*) based restriction fragment length polymorphism (RFLP) genotyping, spoligotyping, and MIRU-VNTR

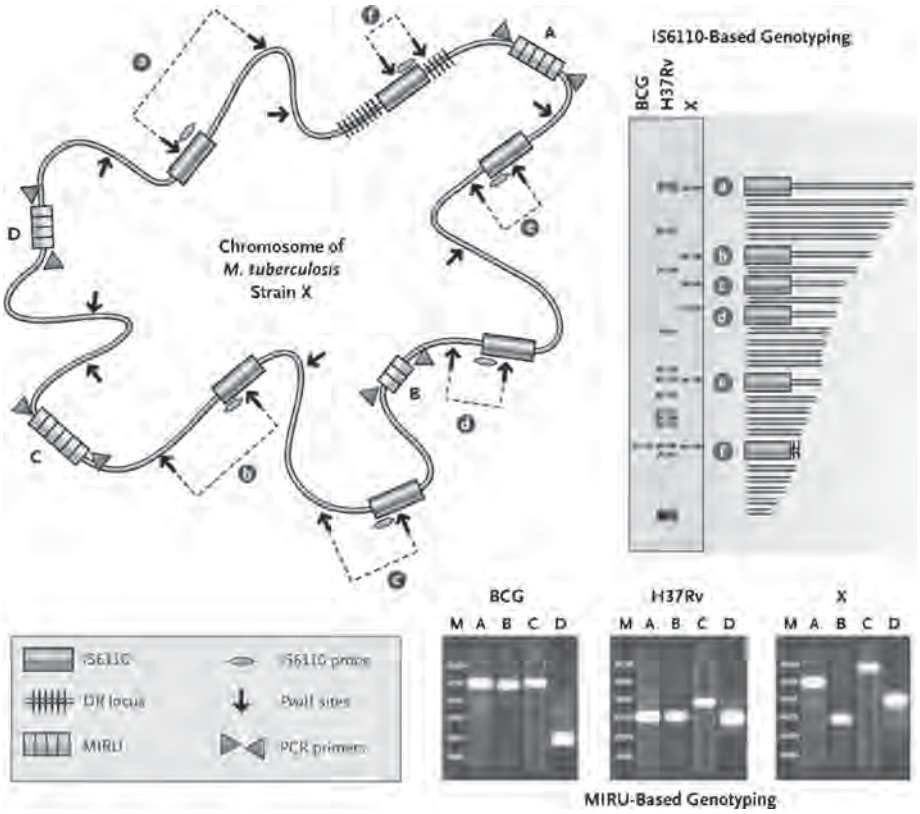


Fig. 1.5. Chromosome of *Mycobacterium tuberculosis* (MTB) hypothetical strain X and genotyping of *M. bovis* bacille Calmette–Guérin (BCG), the MTB laboratory strain H37Rv, and strain X on the basis of IS*6110* insertion sequences and mycobacterial interspersed repetitive units (MIRUs). The top left-hand panel shows the chromosome of hypothetical strain X, as shown by the arrows. The top right-hand panel shows the results of IS*6110*-based genotyping. Mycobacterial DNA is digested with the restriction enzyme *Pvu*II. The IS*6110* probe hybridizes to IS*6110* DNA to the right of the *Pvu*II site in IS*6110*. The size of each hybridizing fragment depends on the distance from this site to the next *Pvu*II site in adjacent DNA (fragments a through f), as reflected by gel electrophoresis of the DNA fragments of BCG, H37Rv, and X. The horizontal lines to the right of the electrophoretic strip indicate the extent of the distribution of fragments in the gel, including *Pvu*II fragments that contain no IS*6110*. The three bottom panels show the results of MIRU-based genotyping. MIRUs contain repeat units, and MIRU analysis involves the use of polymerase chain reaction (PCR) amplification and gel electrophoresis to categorize the number and size of repeats in 12 independent loci, each of which has a unique repeated sequence. The sizes of molecular-weight markers (M) and PCR products for the loci A, B, C, and D in BCG, H37Rv, and X are shown. The specific sizes of the various MIRUs in each strain result in a distinctive fingerprint for the strain (from [10], with permission).

(mycobacterial interspersed repetitive units-variable number tandem repeats) (see below for details of each technique). Figures 1.5 and 1.6 illustrate these three techniques and display the different genetic elements in the MTB genome.

1.3.2.1 IS6110-based RFLP genotyping (See Fig. 1.5)

Until recently, this technique was *the gold standard* approach for genotyping MTB isolates. IS*6110* is an insertion sequence that was identified in the MTBC by Thierry et al. [191]. Through a RFLP analysis, these insertion sequences have been used as epidemiological tools since 1991 [139]. They vary in copy number and may have different integration sites in different strains. From a technical point of view, extracted DNA from a bacterial culture is digested with the restriction endonuclease *Pvu-II*. DNA fragments are then separated according to their molecular weight by gel electrophoresis. The gel is then transferred and hybridized by a specific probe of IS*6110* elements, resulting in easily readable band patterns. The three strains presented in Figure 1.5 (BCG, H37Rv, and X) differ in the number of bands corresponding to the number of IS*6110* copies in the genome and the location of the bands. The protocol of this technique is well standardized, providing results that are comparable between laboratories, and large databases are available (http://www.caontb.rivm.nl/) [81]. Nevertheless, this technique presents several disadvantages. First, it requires culture of MTB and a large amount of DNA. Second, this genotyping method is very time-consuming, labor-intensive, and technically demanding. Third, it has

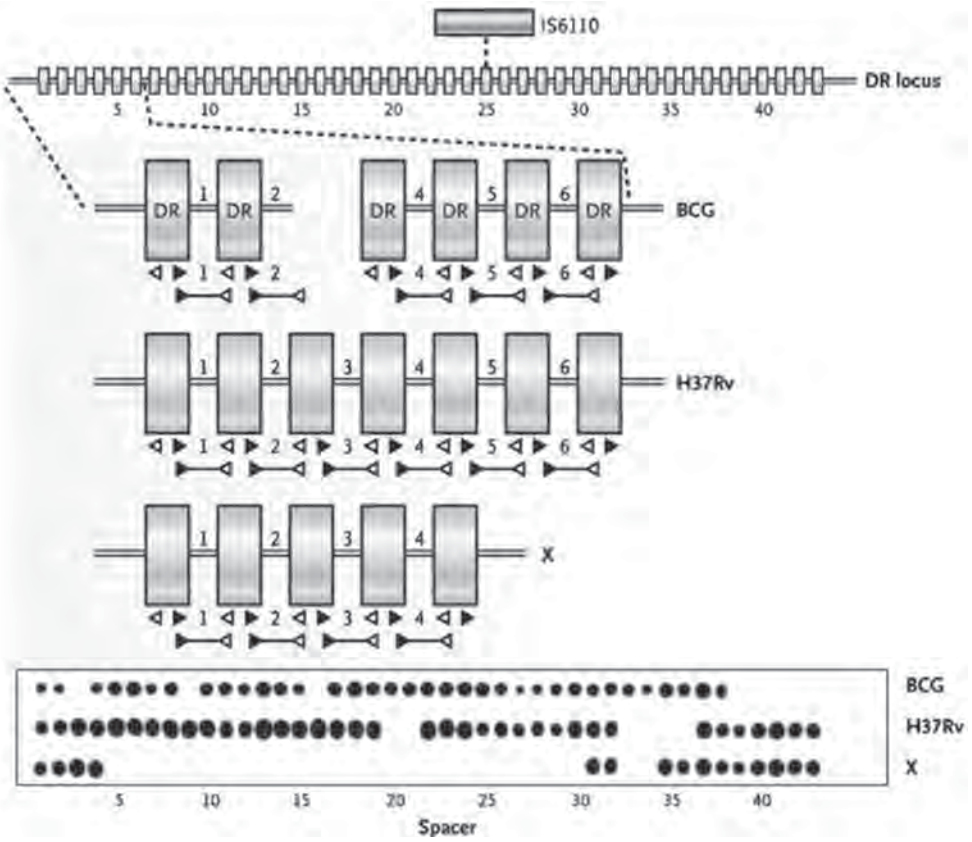


Fig. 1.6. Spoligotyping. The direct-repeat (DR) locus is a chromosomal region that contains 10–50 copies of a 36-bp direct repeat, separated by spacer DNA with various sequences, each of which is 37–41 bp. A copy of IS*6110* is inserted within a 36-bp direct repeat in the middle of the DR locus in most strains. *Mycobacterium tuberculosis* strains have the same overall arrangement of spacers but differ in terms of the presence or absence of specific spacers. Spacer oligonucleotide typing (spoligotyping) involves polymerase chain reaction (PCR) amplification of the DR locus, followed by hybridization of the labeled PCR products to a membrane that contains covalently bound oligonucleotides corresponding to each of 43 spacers. Individual strains have positive or negative signals for each spacer. The top section shows the 43 direct repeats (rectangles) and spacers (horizontal lines) used in spoligotyping. The middle section shows the products of PCR amplification of spacers 1 through 6 of *M. bovis* bacilli Calmette–Guérin (BCG), *M. tuberculosis* strain H37Rv, and *M. tuberculosis* hypothetical strain X, with the use of primers (white and black arrowheads) at each end of the DR locus. The bottom section shows the spoligotypes of the three strains (from [10], with permission).

relatively poor discriminatory power for isolates with fewer than five copies of IS*6110*.

1.3.2.2 Spoligotyping (spacer oligonucleotide typing) (See Fig. 1.6) This method is based on polymorphism of the chromosomal DR (direct repeat) locus. The DR elements, identified by Hermans et al. [88], contain multiple, well-conserved 36-bp DRs interspersed with nonrepetitive spacer sequences (34–41 bp long). Strains vary in the number of DRs and in the presence or absence of particular spacers. Indeed, the spacer oligonucleotide typing (spoligotyping) method described by Kamerbeek et al. [94] detects the presence or absence of spacers of known sequence in an isolate in two steps. PCR is used to amplify the spacers between the DRs. The reverse primer used in the PCR is biotin-labeled, so that all reverse strands synthesized are labeled. Individual spacers are then detected by hybridization of the biotin-labeled PCR product to a membrane on which 43 oligonucleotides derived from spacers of *M. bovis* BCG and MTB H37Rv have been covalently linked (see example in Fig. 1.6): 29 oligonucleotides are from spacers common for BCG and H37Rv, six are from spacers specific to *M. bovis*, and eight are from spacers specific to H37Rv.

Contrary to the IS*6110* genotyping method, spoligotyping is a technique based on polymerase chain reaction (PCR). The method is simple, rapid, and robust, and only small amounts of DNA are needed. It can be done on clinical samples or on strains shortly after inoculation into liquid culture [94]. The results can be represented as a binary code (0 corresponding to absence, 1 to presence) and can be expressed in a digital

format [39], which makes it easy to compare the data between laboratories and with data deposited in the international spoligotyping database SpolDB3 housed at the Pasteur Institute in Guadeloupe [60]. This database is available online at http://www.pasteur-guadeloupe.fr/tb/spoldb3/, although its recent version, SpolDB4, is not yet available. This technique is useful not only to identify the species of the MTBC responsible for the infection, but also to characterize the MTB family at an intraspecific level [205]. The disadvantage of this method is its paucity of discrimination, resulting in the need for another method for resolvent genotyping [169,215]. Second-generation spoligotyping that is more resolvent was recently developed and can detect the presence of the 43 traditional spacers, as well as 51 novel spacers [202].

1.3.2.3 MIRU-VNTR (Mycobacterial interspersed repetitive units—variable number tandem repeat) (See Fig. 1.5) Recently, a new technique was elaborated based on specific repetitive elements of *Mycobacterium tuberculosis* (see Fig. 1.5). Indeed, in 1997 and 1998, novel intergenic repetitive units dispersed throughout the mycobacterial chromosome have been identified and called mycobacterial interspersed repetitive units (MIRUs) by Supply et al. and variable number of tandem repeats (VNTRs) by Frothingham et al. [68,182]. These structures are composed of 40–100-bp repetitive sequences organized in direct tandem repeats that are scattered in several locations throughout the chromosome of MTB H37Rv [183]. The total number of MIRUs or VNTRs is estimated to be about 40–50 per genome (41 loci are present in MTB H37Rv [183]). These structures are comparable to minisatellites observed in higher eukaryotes [118]. Some of these MIRU-VNTR loci have been tested and have shown their usefulness for molecular epidemiology studies [68,118]. The sequencing of MIRUs loci identified 12 of them, displaying variations in tandem repeat copy numbers as well as sequence variations between repeats [183]. Mazars et al. [118] published a PCR-based typing method by using these 12 loci for molecular epidemiology studies, and this technique has already shown its potential to discriminate between MTB strains in different studies (see Fig. 1.7).

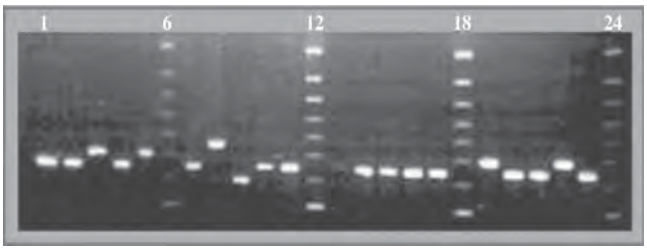


Fig. 1.7. Patterns of MIRU-VNTR of *Mycobacterium tuberculosis*. Lanes 1–5, 7–11, 13–17, and 19–23: Patient's pulmonary isolates from Montpellier Hospital, France. Lanes 6, 12, 18, and 24: Molecular size markers (Fraisse, unpublished data, all rights reserved).

1.3.3 How Should the Most Appropriate Molecular Marker be Chosen?

Genetic typing is the means by which the microbiologist is able to discriminate and catalogue microbial nucleic acid molecules. As said above, the diversity among nucleic acid molecules provides the basic information for all fields. Nevertheless, currently, full-genome sequences for multiple isolates are rare, implying that genetic typing is still done by methods that are inherently suboptimal [201]. The first fundamental characteristic to define a genetic marker as a good molecular tool is the portability of methodology between laboratories. Van Belkum et al. [201] noted that communication of data can be obstructed because of a general lack of standardized genetic typing procedures and thus, except for primary DNA sequences, typing data frequently suffer from limited interlaboratory reproducibility. Molecular marker standardization is undoubtedly essential for all research fields, whether medical, epidemiological, or genetics. Optimal typability, a high degree of reproducibility, adequate stability, and unprecedented resolving power must characterize the gold standard typing technique. In addition, the procedures should not be too expansive or complex and should be easily accessible. Furthermore, setting up large databases is undoubtedly an advantage for international epidemiological surveillance and for free and easy exchanges between laboratories.

Another important point raised by Van Belkum et al. [201] is that the technique should be chosen with care to provide answers to a specific question. For example, currently, techniques based on nucleic acid polymorphism are more frequently applied and better appreciated than the phenotypic methods in taxonomy, epidemiology, and evolutionary studies. Furthermore, the choice of the optimal molecular markers in accordance with the scope of the study also depends on the space and time scales in which the data were collected or explored. Tibayrenc [192] defined three different time and space scales: (i) days to months, the hospital or village, referred to as short-term epidemiology; (ii) months to years, country- or continent-wide, up to the entire geographical range of the species, referred to as long-term epidemiology; and (iii) millions of years, country- or continent-wide, up to the entire geographical range of the species, such as in phylogenetic studies. Here, Tibayrenc [192] pointed out a central notion, the *speed of evolution* (*molecular clock*) of a given marker, which conditions its power of resolution. Fast markers allow conducting short-term epidemiology studies, while slower markers are more appropriate for long-term epidemiology, and slow markers such as ribosomal RNA genes are more appropriate for phylogenetic studies. Nevertheless, the resolution power of each marker is a function of the organism and the species under study. For MTB, it appears that the gene typing as used by Sreevatsan et al. [177] and Musser et al. [130] could be useful for phylogenetic studies, considering of course polymorphic genes. While the three techniques are based on repetitive chromosomal elements, IS*6110*, spoligotyping, and MIRU-VNTR are better adapted for molecular epidemiology, there is not a single best marker. From the various comparisons of these three markers [86,98,99,115,118], from a resolution power point of view, IS*6110*-based RFLP typing

and MIRU-VNTR appeared very appropriate for short-term epidemiology studies, whereas spoligotyping is more suitable for long-term epidemiology studies. Nevertheless, several studies showed clearly that using multiple methods for molecular epidemiology is necessary [37,132,150,181]. Several authors recommend spoligotyping associated with MIRU-VNTR for molecular epidemiology studies [9,36,40]. This is also developed within the framework of the US national genotyping program to characterize all initial isolates of MTB [40]. One of the limitations is the cost of these techniques, which prevents their routine use in low-income countries.

1.3.4 Population Structure of MTB and Epidemiological Consequences

1.3.4.1 Theoretical and technical assessment

Reproduction is the process by which living creatures transmit their genes to produce another generation of living creatures. This is a common phenomenon to all living organisms but it has a great impact in particular for the population structure of microorganisms, as reproduction strategies are diverse, with a variety of sexual and asexual processes expressed. In bacteria, on a theoretical basis, four different types of population structures have been proposed by Smith et al. [167]; the two extremes being the clonal model on one hand and the sexual model on the other hand. *Clonal or asexual propagation* refers to populations in which the offspring are genetically identical to their parent [195] and thus genetic exchanges are rare or absent (e.g., *Salmonella* [167]). In bacteria, the *sexual model* refers to organisms in which genetic exchanges are very frequent (e.g., *Neisseria gonorrhoeae*; [138]). It is worth noting that in bacteria, the sexual model does not correspond to true sexual reproduction but to frequent exchanges of genetic information (genetic recombination) occurring by the classical bacterial processes such as *transformation*, *conjugation*, *and transduction*. Nevertheless, between these two extremes, Smith et al. [167] described two other intermediate models: cryptic speciation and epidemic clonality. In the case of cryptic speciation, the species under study is subdivided into two or more biological species, each being sexual (e.g., *Rhizobium meliloti*; [167]), but no genetic exchanges occur between the different species. Epidemic clonality is characterized by sudden clonal expansion of a relatively short-lived type occasionally observed for a species that otherwise replicates in a sexual model (e.g., *Neisseria meningitidis*; [167]). Other evolutionary mechanisms, such as migration, selection, and genetic drift, also play a role in the genetic structure of populations, but reproduction is the basic biological process influencing the population structure. Identifying the reproduction system is all the more essential, as it governs the allelic and genetic distribution in natural populations and conditions the stability of genotypes in space and time [193]. Therefore, this has important consequences from an epidemiological and medical point of view (strain typing, pathogenicity, vector specificity, and susceptibility to drugs and vaccines), and hence on the epidemiological and medical relevance of microorganism genotypes. For pathogenic microorganisms, the clarification of population structure provides unique insights into crucial public health issues, such as the appearance and persistence of variants escaping immunity or the emergence of resistance to antibiotics [129,168,176]. Consequently, it appears incontestable that knowledge of the reproductive system is essential to exploiting molecular epidemiological data fully and correctly.

Population genetics is the scientific discipline that studies genetic diversity and its distribution in natural populations and all the biological events influencing the population structure such as the reproduction system. Two kinds of tests are used in order to infer the population structure in samples being investigated. Tibayrenc [193] detailed the theoretical basis of these studies for microorganisms. Briefly, these tests were based on the two main consequences of sexual reproduction: *segregation* of alleles at given loci (reassortment of different alleles at a given locus) and *recombination* of genotypes (reassortment of genotypes at different loci). Segregation tests are related to *Hardy–Weinberg equilibrium* and imperatively require a diploid level of the organism and an identification of alleles. Therefore, these tests are not applicable to bacteria nor to MTB, which has a haploid genome. Recombination tests are related to *linkage disequilibrium* (nonrandom association of genotypes occurring at different independent loci) and contrary to segregation tests, they can be used irrespective of the ploidy level of the organism under study and even without identifying individual alleles and loci [194]. The only requirement for these tests is to use molecular markers that show a sufficient level of polymorphism and make it possible to perform a multilocus analysis (because loci must be independent) (see [193] for details). The MIRU-VNTR technique compared to IS*6110* and spoligotyping techniques shows the necessary properties to be used for population genetics studies: it is a multilocus marker and the loci are distributed independently along the bacterial genome. In contrast, IS*6110*-based RFLP cannot be used to analyze linkage disequilibrium, as they do not reveal the variability of independent genetic loci. Furthermore, spoligotyping cannot be assumed to be independent from IS*6110*-based RFLP, as this locus is a hot spot for IS*6110* insertions, and changes within this region are often caused by IS*6110*-associated events [57,62,81,88,106]. Furthermore, the DNA sequences of multiple *housekeeping genes* can also be used to infer the population structure and the phylogenetic history of bacterial species. Nevertheless, as polymorphic genes should be selected to conduct these studies, it is worth noting that the choice is limited in MTB because there is an extremely limited amount of unselected nucleotide sequence variation in structural genes and housekeeping genes in this bacteria [63,130,177].

Another discipline, molecular phylogenetics, also appears vital for understanding evolutionary molecular biology and molecular epidemiology. This discipline is devoted to understanding the hierarchical structure of biological diversity through genetic data. One important outgrowth of the phylogenetic revolution is the recognition that phylogenetic trees

provide an important and appropriate context to address questions in a variety of disciplines such as molecular epidemiology and evolutionary biology. More and more, the phylogenetic approach is used to explore the population structure and to infer the system of reproduction of various organisms [58,83]. These analyses contribute complementary information beyond population genetics studies, such as genetic structuring in a population, identification of a genetically individualized entity, for example, cryptic species and epidemics. Furthermore, the congruence or incongruence of different gene phylogenies also provides substantial insight into the population structure. Indeed, congruence of several independent genes is evidence of a lack of genetic exchange, whereas phylogeny incongruence reflects frequent genetic exchange.

1.3.4.2 Population structure of MTB Mycobacteria, like other bacteria, may have the potential to exchange DNA. Indeed, experimental transduction has been performed in MTB [85], and natural conjugation has been demonstrated for *M. smegmatis* [143]. Nontuberculous mycobacteria can acquire antibiotic resistance genes from other species [80,140]. Furthermore, simultaneous infection of patients by two different strains was evidenced in high-incidence areas [16,27,218]. All these data suggest that MTB could be able to exchange DNA in natural populations. Nevertheless, authors have hypothesized for several years that this species has a clonal population structure. This statement was based on the preponderance of certain genotypes and on the low level of genetic polymorphism and not on a rigorous population genetics analysis. Contrary to a widespread idea, the restricted gene sequence diversity and empirical observation of some predominant genotypes in various epidemiological studies provide no indication of its population structure, as they are compatible with distinct population structures with variable levels of recombination [58,59,167,176]. Furthermore, until the year 2000, no marker presented the necessary properties (i.e., a polymorphic marker based on several independent loci) to conduct population genetic studies (see above). The development of the MIRU-VNTR technique and the sequencing of several MTB strains finally provided appropriate methodologies for studying population structure and thus MTB's mode of reproduction. Consequently, few studies based on these markers supported the conclusion that *MTB is a clonal organism, with no evidence of lateral gene transfer* [7,184,187]. Two studies conducted in South African and Moroccan populations tested linkage disequilibrium by means of MIRU-VNTR techniques [6,175]. A third study was mainly based on a phylogenetic analysis of polymorphic gene sequences of a sample of 316 UK clinical isolates [178]. Despite the strong linkage disequilibrium observed in these populations and consequently the relevant identification of the typically clonal evolutionary model, *the occurrence and significance of genetic exchanges within natural populations of this species remain to be demonstrated*.

Recently, a study based on phylogenetic and sequence analysis was published by Gutierrez et al. [83] in order to unravel the evolutionary success of MTB. Members of the MTBC suggested representing the clonal progeny of a single successful ancestor, resulting from a recent evolutionary bottleneck that occurred 20,000–35,000 years ago [177]. Gutierrez et al. [83] identified the progenitor of MTBC, which includes *M. canettii* (already suggested by Brosch et al. [20]), a rare tubercle bacillus with an unusual smooth colony phenotype [199], and other smooth tubercle bacilli from Djibouti. These authors proposed to call this group of strains *M. prototuberculosis* species. From a population structure point of view, the interesting element in this paper is the observation of a mosaic structure of some genes and an incongruence of gene phylogenies. Both results suggested that DNA recombination is frequent in this population [83]. In contrast, using the same analysis, they detected no evidence of recombination among the MTBC strains, consistent with the previously reported clonal population structure. Furthermore, results supported that despite its present clonal and highly conserved structure, MTBC is actually a composite assembly of genetic sequences resulting from multiple remote horizontal gene transfer events. Therefore, the authors proposed several potential explanations for the apparent absence of recombination among the MTBC strains after the bottleneck [83]: (i) the MTBC strains could have lost the capacity of horizontal gene transfer, (ii) horizontal gene transfer events are too rare among tubercle bacilli to have occurred since the MTBC bottleneck, and (iii) the MTBC ecological niche differs from that of *M. prototuberculosis* and offers no opportunity for recombination events. Thus, further progress in the understanding of evolutionary biology of MTBC and MTB still requires deciphering why MTB is no longer able to exchange genetic information in natural populations and whether the different species, families, and populations belonging to MTBC present all the same population structure.

In summary, because of the strong linkage disequilibrium and the phylogenetic studies developed in several populations, it appears that MTB follows a typical clonal model. This implies that MTB genotypes can be considered as epidemiologically discrete units of research, which Tibayrenc [192] calls discrete typing units (DTUs), and thus can be used as markers for applied studies (epidemiological tracking, vaccine and drug design, clinical studies). From these clonal characteristics, these DTUs or MTB clones can be specifically identified by appropriate genetic markers or "tags" [192]. Nevertheless, the description of a MTB progenitor and of the high frequency of genetic exchanges in this ancestral lineage does not allow excluding the possibility of genetic exchanges in MTB.

1.4 USE OF MOLECULAR EPIDEMIOLOGY FOR UNDERSTANDING TUBERCULOSIS TRANSMISSION AND PATHOGENESIS

The primary goals of TB control at the community and individual levels are to identify the bacteria responsible for infection and to treat infected people. Nevertheless, it is essential to control and fight the disease by tracking the strains identified

as the source of infection and thus discriminating strains. From this crucial need *molecular epidemiology* was born, which has become a major field of research in MTB in the last 20 years. This scientific domain corresponds to the interpretation of molecular data through the conventional epidemiologic studies. Thus, this domain involves several disciplines, encompassing medicine, molecular biology, epidemiology, and biostatistics. Molecular epidemiology is now largely recognized as a science that makes it possible to understand the transmission, pathogenesis, and etiology of human disease [40,188]. This discipline provides tools for clinicians, microbiologists, and epidemiologists for investigating infections. Indeed, molecular epidemiology is essential to studying the spread of MTB in *epidemics* and *outbreaks*, to analyzing the transmission dynamics, and to determining the risk factors for TB transmission in a community. It plays a great role in distinguishing between *exogenous infection* or *reinfection* and *endogenous reactivation*. In the laboratory, it can also be used to identify cross-contamination. In addition, molecular tools have provided markers able to identify specific gene mutations corresponding to various drug resistances [121,147,189]. Genotyping determines whether the development of drug resistance in a TB patient during treatment is caused by the same strain or another strain by exogenous reinfection. In regard to virulence and pathogenesis studies, molecular epidemiology has already proven to be relevant to attaining insight into the strain's capacity to be pathogenic or drug resistant. Finally, this discipline becomes fundamental to developing strategies for treatment and prevention of diseases. Therefore, it is worth noting that for interpretation of molecular epidemiology results, it is important to consider not only all the clinical, biological, and epidemiological data recorded from tuberculous patients (requiring interview and biological analysis) but also phenotypical, biological, and epidemiological data concerning MTB isolates (requiring an antibiogram, genotyping, and culture).

Within this framework, this section is a review of various epidemiological issues for which molecular epidemiology can improve the understanding of MTB transmission and pathogenesis.

1.4.1 MTB Families and Worldwide Distribution

The world has entered an era of "*diseases without borders*," with 1 million people crossing borders daily, too often carrying with them diseases that were once geographically isolated. By virtue of its worldwide distribution, TB, like HIV, is classified in this category. Lazcano-Ponce et al. [104], assert that the *framework is essential for collaboration on alerting the world to epidemics and responding to public health emergencies*. This is necessary to guarantee a high level of security against the dissemination of communicable diseases in an ever more globalized world. Thus, global molecular epidemiology studies of MTB appear as fundamental as local ones in order to develop strategy to control and fight TB.

As described in the previous section of this chapter, genotyping allows tracking of MTB strains at local as well as global levels. Genetic data allow identifying and following the spread of a particular genotype worldwide. For greater convenience, MTB species have been subdivided into *families*, also called clades in the literature, corresponding to specific genotypes or *clusters* (a cluster corresponds to a particular genotype shared by two or more MTB isolates) or groups of genotypes (corresponding to the DTUs described by Tibayrenc [192]; see above). These families or clades appeared from the mid-1990s with the worldwide development and technological progresses of molecular epidemiology studies. The major families, or those that have been studied more thoroughly, bear a specific name. As an example, we can describe in detail the case of the best-known family, *the Beijing family*, which was first described by Van Soolingen et al. in 1993 [208]. These researchers identified this family by analyzing the population structure of MTB strains from the Republic of China. The vast majority of strains under study belonged to a genetically closely related group that originated from the province of Beijing; therefore, they designated this group the Beijing family. They observed that strains of this family were also found to dominate in neighboring countries such as Mongolia, South Korea, and Thailand, whereas a low prevalence of such strains was observed in countries on other continents. From these data, they suggested that strains of the Beijing family recently expanded from a single ancestor that had a selective advantage.

To date, the most recent global study has been conducted by Filliol et al. [61] on a data set of 13,008 isolates from more than 90 countries. This study, based on the spoligotyping technique, updated the data published by Soini et al. and Sola et al. [170–172]. All the results were integrated into the SpolDB3.0 database. They identified 813 different spoligotypes shared by 2 or more isolates, which contained 11,708 isolates, whereas 1300 spoligotypes were orphans. They evidenced seven major MTB families, the Beijing family, the EAI family (East African-Indian), the CAS family (Central Asian), the T group of families, the Haarlem family, the X family, and the LAM family (Latin American and Mediterranean). The Beijing type was predominant (see Fig. 1.8), followed by the Haarlem type, then by the X types, which are highly prevalent in the United Kingdom and the United States. Nevertheless, Filliol et al. [61] underlined major differences in MTB populations between the subcontinents under study. The global observation was able to define that most MTBs are confined to specific geographic locations [40,56,61]. Nevertheless, these worldwide studies and the numerous molecular studies already published showed that some families are widely dispersed both geographically and temporally, suggesting that they are more transmissible, or more pathogenic than other strains [40,61]. Daley [40] described the Beijing family as detected in high proportions among the strains in several countries (Fig. 1.8) and as associated with large outbreaks, febrile response, treatment failure, relapse, and drug resistance. But to date, it is not clear why the Beijing family strains are so widely disseminated [12]. Daley [40] suggested different hypotheses such as a selective advantage of

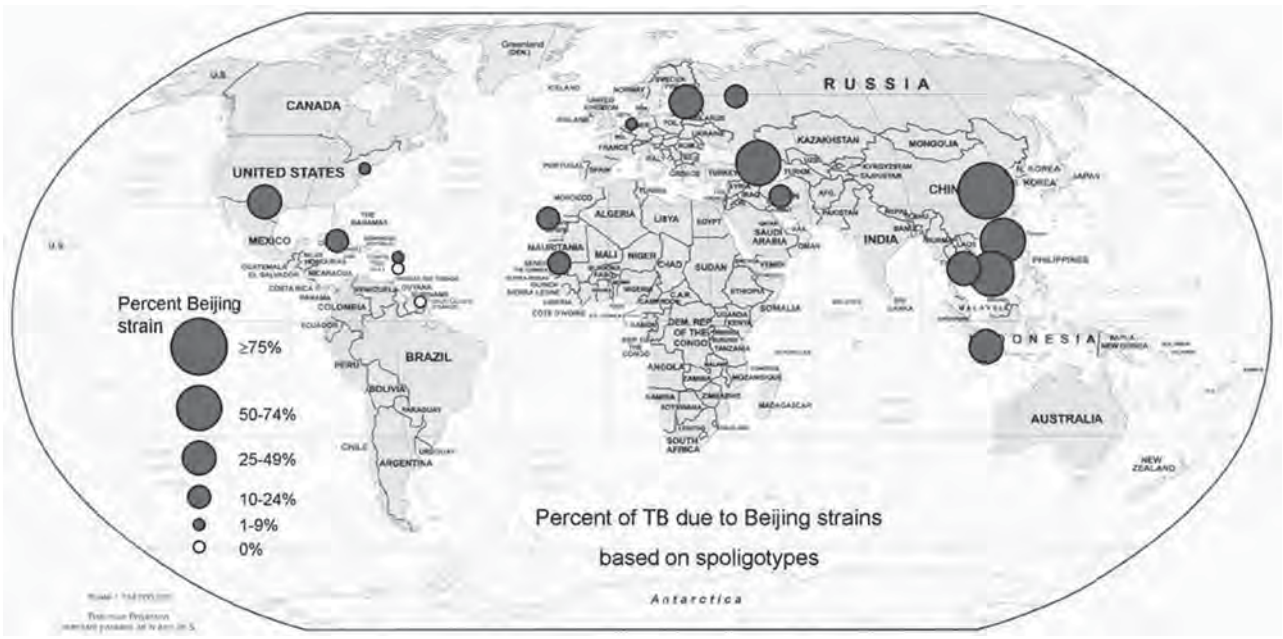


Fig. 1.8. Percentage of tuberculosis due to Beijing strains. Data from studies based on spoligotyping (from Glynn et al. [77]).

these ubiquitous families, a better ability to establish infection, a more rapid progression from infection to disease [10,112], and a longer time to spread. Further research is needed in order to determine why some families are more widespread than others.

1.4.2 MTB in Developing Versus Developed Countries

As we have seen above, *tuberculosis has disseminated globally, but it is not distributed equally throughout the world, with developing countries having by far the highest burden*. Indeed, more than 90% of TB cases occur in developing countries (see Fig. 1.1). The areas most hard hit by this disease are Africa, Southeast Asia, and Eastern Europe. Sub-Saharan Africa has the highest incidence (290 per 100,000 population) with more than 1.5 million cases of TB. The most populous countries of Asia have the highest numbers of TB cases: India, China, Bangladesh, and Pakistan together account for more than half of the global burden. Case numbers have declined more or less steadily in Western and Central Europe, in North and South America, and in the Middle East. These data evidence once again the inequality between developing and industrialized countries in our modern society. As Lazcano-Ponce et al. [104] explained in their paper, investment and investigation in health also involve inequalities at the global level, and this includes insufficient north–south transfer of funds, technology, and expertise in the health field, including the specific area of communicable diseases. Furthermore, although lower-resource countries have by far the highest burden of TB, we can regret that molecular epidemiology studies have not yet been conducted in many of these countries.

Nevertheless, global studies showed that TB transmission varies greatly depending not only on the country but also on the country's development level. The distribution of each MTB family and the number of orphans change geographically (see Filliol et al. [61] for details), for example, the number of orphan types (or singletons) ranged from a low of 8% (North America) to a high of 21% (Middle East and Central Asia); the Beijing family ranged from 2% in South America to 3–5% in Central America, Europe, Africa, and the Middle East and Central Asia, 13% in Oceania, 16% in North America, and as high as 45% in East Asia. Daley [40] noted that considering the ability of the Beijing family to become multidrug resistant, its high prevalence in certain regions of the world is an important issue for effective TB control.

Besides the MTB family distribution, genetic diversity is also an important indicator of TB endemicity and transmission, as well as the efficiency of TB control [10]. MTB's genetic diversity differed greatly in developing versus developed countries [87]. Indeed, low genetic diversity within MTB populations is typical of a high TB incidence or of an epidemic pattern and suggests inadequate TB control. This situation is encountered more particularly in developing countries with a high TB incidence. Some studies evidenced the slight genetic polymorphism in different regions such as Honduras, Ethiopia, Tunisia, different countries of West Asia, and the Southern Africa region [87,145,184,209]. A low genetic polymorphism was also observed in the case of localized epidemics characterized by a rapid spread of particular strains in specific areas (called hotspots). These phenomena can be observed in developing countries as well as in developed countries. A number of examples can be cited: the

particular case of the spread of multidrug-resistant isolates belonging to the W-Beijing family in a Russian prison [197] and the multiple occurrence of MTB epidemics in New York City (see review by Paolo and Nosanchuk [141]).

In contrast, in regions with low TB incidence, mostly in developed countries, genetic diversity is higher, as in Denmark and the Netherlands where most isolates show unique DNA fingerprint patterns [87,216]. This is also true when considering a more restricted geographic area such as cities. Indeed, a retrospective study (2002, 2003) concerning isolates from Montpellier, France, showed, by means of MIRU-VNTR and spoligotype techniques, a high level of genetic polymorphism and weak clustering (Fraisse et al. unpublished data). These results suggest efficient control of TB, which prevents the spread of MTB strains in populations and a higher rate of reactivation compared to recent transmission.

However, unexpected results were obtained for several countries with high TB incidence. For example, studies conducted on samples isolated in Morocco and Burkina Faso showed higher values of genetic diversity (Tazi et al. [187]; Godreuil et al., unpublished data). Further studies are necessary in order to understand the epidemiological significance of these data.

1.4.3 Clinical and Epidemiological Relevance of Molecular Epidemiology at the Local Level

Routine public health investigations do not allow deciphering the chain of transmission, and the source of infection and the characteristics of strains responsible for infection are unknown. *Molecular epidemiology by genotyping with resolvent markers can fill this gap concerning the chain of TB transmission*. Indeed, isolates from patients who were infected by a common source or belonged to the same chain of transmission have identical or closely related genotypes (considering genotyping with the most resolvent markers); in other words, clustering is assumed to reflect recent transmission within a population [150,164]. In contrast, MTB isolates from patients with epidemiologically unrelated TB present a broad variability of genotypes. For example, it has been estimated on the basis of clustering of DNA fingerprint patterns, that half of TB cases in a South African mine hospital were caused by ongoing transmission [78].

At the local level, it is therefore important to link molecular epidemiology and classical epidemiological tools in order to identify contacts of patients outside the home and workplace and in the locations where they spend time. The patient's environment could thus be screened for TB infection and disease and contacts treated if necessary. For example, Torrea et al. [196] identified several chains of transmission (familial or geographical cases) in French Polynesia using a detailed molecular study. Nevertheless, Daley [40] explained on the basis of several studies [164,203] that a relatively small proportion of TB cases presenting identical genotypes were named as a contact by the source case [41]. This may be attributable to unsuspected transmission not easily detected by conventional contact tracing investigations. Indeed, transmission can occur through only short and casual contact, difficult to pinpoint [207].

Furthermore, *molecular fingerprinting can be used at a local level to establish or rule out the existence of an emerging outbreak*. The investigation of outbreaks remains central to the control of TB. For example, Diel et al. [48] described an ongoing outbreak in the Federal State of Hamburg, Germany, by a molecular epidemiology study. They identified various infectious chains of contact that, starting in a bar that played the role of a turntable, moved out rapidly into several areas such as housing for homeless men and alcoholics and a tank-cleaning firm. This study can be considered as a model because it combines detailed clinical and epidemiological data and phenotypical and molecular studies. Nowadays, numerous outbreaks continue to be identified in various public areas such as hospitals, schools, bars, prisons, nursing homes, and homeless shelters in developed and developing countries [38,52,90,92,105,116,120,153,159,178]. These studies were also able to identify risk factors for TB transmission. It is worth noting that *MDR and HIV are often incriminated in the emergence of outbreaks* [67,100]. It has been observed that MDR strains are less responsive to standard therapy, and patients remain infectious for longer periods of time. Breathnach et al. [18] noted that the outbreaks linked to HIV are globally described in hospitals where AIDS patients are cared for together, and increasingly involve MDR strains (see below for more details on HIV and MDR linked to TB).

At a nosocomial level, in addition to detection of outbreaks, genotyping of isolates from patients is also useful for identifying cross-contamination and mixed infection, as well as for differentiating reactivation from reinfection. Barnes et al. [10] evaluated that 3% of patients whose cultures are positive for MTB in clinical laboratories do not have TB. Cross-contamination is suspected because these patients present with negative acid-fast smears and clinical findings. Comparing the isolate genotype with those circulating in the laboratory makes it possible to identify cross-contamination and thus stop unnecessary anti-tuberculous medication. Genotyping can also evidence cases of multiple infections. The occurrence of mixed infection is now widely accepted, whereas until recently it has been assumed that patients could be infected only with a single MTB strain, and infection with one strain is thought to confer immunity to MTB superinfections [161]. Several molecular investigations showed either simultaneous infection with multiple MTB strains [16,161] or multiple infections caused by an exogenous reinfection [27,161]. Furthermore, these mixed-strain infections can involve drug-sensitive and MDR strains [8,190].

Molecular fingerprinting appears to be useful for differentiating (*i*) *a reactivation of latent infection from a recent infection and* (*ii*) *a relapse with the previous MTB strain from an exogenous reinfection by a new strain*. As described in Section 1.2 of this chapter, the first episode of an active case of TB can be caused by either a recent transmission of MTB strains or a reactivation of latent infection. Isolates that have the same molecular fingerprint are presumed to be part of a cluster of recent transmission, with one or more people in the cluster having transmitted infection

to the others [75,212]. For example, a recent report described a recent infection of MTB in northern Malawi, in which 72% of the strains were clustered [76]. Reactivation of latent infection occurs in about 10% of infected individuals, leading to active and contagious tuberculosis [89,113,114]. It has been demonstrated that reactivation of latent infection contributes substantially to the incidence of adult TB, especially in more developed countries where disease prevalence is fairly low. As examples, (i) Geng et al. [71] obtained data that suggested that in the United States among foreign-born people, TB is largely caused by reactivation of latent infection, whereas among US-born individuals, many cases result from recent transmission and (ii) Lillebaeck et al. [110] presented molecular evidence of *reactivation of MTB 33 years after primary infection*.

Concerning the cases of a second TB episode, in their review Chiang and Riley [28] detailed the debate that has existed for decades concerning reactivation and reinfection because reinfection was considered to be an uncommon cause of TB. Classically, before genotyping development, relapses were associated with reactivation of MTB infection. *Several molecular studies showed clearly that reinfection causes a significant proportion of recurrent TB episodes* [24,45,206]. This proportion seems to vary as a function of the area, the endemicity, and the biological status of patients [45]. Nevertheless, Lambert et al. [102] reported that the importance of reinfection remains unclear because only very few studies are adequately designed for that particular research objective and/or report a sufficient number of observations. They consider that only the study published by Sonnenberg et al. [174] provides an exact estimate of the incidence of recurrence due to reinfection, indicating its importance in HIV-infected patients in an environment with an unusually high TB incidence.

1.4.4 Use of Genotyping to Study the Impact of HIV/AIDS and Drug Resistance on Pathogenesis and Transmission

As described above, TB incidence is linked to poverty and poor living conditions, in some cases to civil conflicts and wars, to deteriorating health services, and to lack of drug availability. Besides these social factors, two major problems regarding the control of TB are emerging: coinfection with HIV and resistance of MTB to the currently used regimen of tuberculostatics. We can approach the problem of HIV and drug resistance together, as it has been demonstrated that they are strongly associated [11,51,55,125,127,165]. Agerton et al. [1] also described outbreaks of MDR-TB involving hundreds of cases, many of whom were infected with HIV, with high mortality rates [13,33,67,93,200].

DNA fingerprinting can determine that in many of these outbreaks, the susceptibility of HIV-positive patients to tuberculosis infections and the accelerated breakdown to disease often result in more rapid transmission of the infection [42,50,55,149,153,165]. Furthermore, the study of MTB isolates obtained from AIDS patients by fingerprinting showed that reinfection and relapse both occur in HIV-infected patients, as the susceptibility to superinfections will most likely be related to the immune status of the patient. By combining classic and molecular epidemiology, Sonnenberg et al. [174] showed that HIV-1 infection is a risk factor for recurrence, as HIV-1 is strongly associated with disease caused by reinfection but not with relapse.

From an evolutionary point of view, since the development of detailed fingerprinting of MTB strains, the genetic divergence of strains circulating in HIV-positive and HIV-negative patients has been debated. As stated by Ahmed and Hasnain [3], it has been speculated that HIV/AIDS patients constitute an ecological niche for MTB, where less virulent strains multiply freely without the selection pressure provided by an optimal immune response. One study has been conducted where significantly different genotypes were observed for HIV-associated tubercle bacilli as compared to bacilli recovered from HIV-uninfected patients [2]. In contrast, Yang et al. [217] obtained results that suggested an equal risk of infection with a defined MTB clone for HIV-seropositive and HIV-seronegative individuals. These latter results were also confirmed by a recent study on a MTB population from Burkina-Faso (Godreuil et al., unpublished data).

With drug-resistant strains, genotyping determines whether the treatment failure and the development of drug resistance are caused by the same strain (inadequate treatment) or a new strain (reinfection during treatment). Several studies have reported that the development of drug resistance may be associated with either the same or different strains [78,79,165,206,211]. The relative contribution of these different mechanisms to treatment failure and/or the development of drug resistance seems to vary according to the populations studied. Nevertheless, Sonnenberg et al. [174] demonstrated that even in a setting with high rates of TB transmission and HIV-1 infection, the dominant mechanism of drug resistance while on treatment was acquisition rather than transmission. Thus, despite reinfection being a possible mechanism of treatment failure and the development of drug resistance, it appears uncommon in comparison with the number of patients who had acquired drug resistance with the same strain.

Furthermore, molecular studies identify whether drug-resistant strains are significant risk factors for secondary cases and thus for outbreaks. Daley [40] found that several molecular epidemiological studies have reported that patients who have drug-resistant strains were less likely to cluster, suggesting that drug-resistant strains might be less prone to being transmitted or to causing active disease [69,78,210]. Burgos and Pym [21] have also recently reported that isoniazid-resistant strains confer a significantly lower number of secondary cases than drug-susceptible strains. Daley [40] concluded that these findings support the hypothesis that drug-resistant strains are less likely to cause disease than drug-susceptible strains. Nevertheless, different environmental or biological conditions counterbalance this weak potential for being transmitted and to causing active disease. First, Post et al. [146] estimated that 8–35% of patients with MDR-TB have persistently active disease that is refractory to a multidrug

regimen [156,160,180,185] and thus are a constant source of transmission of MDR-TB [67,151,160,206]. Second, Daley [40] reported that there are some populations in which drug resistance is neither detected nor treated effectively and where the longer-duration regimens might offset the bacterium's diminished capacity to cause secondary cases [21]. Third, Daley [40] explained that in areas that have high prevalence rates of HIV, the increased host susceptibility, even to strains that have diminished virulence, may also offset bacterial difference [128]. Fourth, a review of the literature concerning drug-resistant TB and especially MDR-TB showed that only a few clones are mainly responsible of MDR-TB outbreaks and thus would have a higher virulence and a higher capacity to be transmitted and to cause disease. The most frequently cited example is the Beijing/W type, which is described worldwide and is involved in numerous MDR-TB outbreaks [74,103,126,128,206].

From an experimental point of view, it has also been demonstrated by several authors that drug-resistant strains are less virulent and present a decrease in pathogenicity in comparison to drug-sensitive strains [29,122,152]. Indeed, Meacci et al. [121] exposed that drug-resistant bacteria are believed to grow more slowly than susceptible bacteria, as mutations conferring resistance reduce their overall fitness, a phenomenon known as cost of resistance [107]. Nevertheless, as described above, the emergence of MDR MTB strains is alarming and is a worldwide health care problem, thus contradicting most experimental data. Several authors, however, have demonstrated in other bacteria that fit variants are quickly selected in a drug-resistant bacterial population, in which compensatory mutations eliminate the biological cost of resistance [4,15,108,175]. Meacci et al. [121] demonstrated by following up a tuberculous patient with active disease for more than 12 years that phenotypic and genotypic changes occurred in the drug resistance of MTB isolates. First, molecular typing showed a single parental strain that infected the patient and persisted throughout the disease. Second, molecular analysis of the drug-resistance-related genes revealed that discrete subpopulations evolved over time from the parental strain by acquiring and accumulating resistance-conferring mutations to isoniazid, rifampin, and streptomycin. Overall, authors noted that during a chronic infection, several subpopulations may coexist in the same patient with different drug susceptibility profiles [121]. This was also observed by Post et al. [146] in a population of 13 HIV-negative patients with MDR-TB that was refractory to chemotherapy given for 12 months. Meacci et al. [121] described the emergence of a successful MDR-TB strain during the genetic and phenotypic changes, resulting from progressive accumulation of genetic alterations, possibly conferring a selective advantage for bacterial survival. Low compliance with therapy may have elicited the selection of resistant strains, which also persisted after stopping treatment. These evolutionary changes could partly explain the numerous outbreaks of peculiar drug-resistant strains recovering an increased potential for being transmitted and causing disease.

1.5 URGENT NEEDS FOR TB CONTROL, LIMITATIONS, AND NEW ISSUES FOR MOLECULAR EPIDEMIOLOGY

This section aims to define the urgent needs for improving TB control and to detail the limitations of modern molecular epidemiology studies. Indeed, molecular epidemiology approaches still present drawbacks that need to be resolved in order to advance the knowledge on TB transmission and enable better public health control strategies. This section will also include the description of molecular technologies that promise to improve molecular epidemiology studies. All the molecular methods described here are not particularly recent, but they are not used routinely for MTB and seem promising for MTB molecular epidemiology.

1.5.1 Urgent Needs for TB Control and Molecular Epidemiology

The development of DNA fingerprinting and molecular epidemiology has pushed forward our understanding of MTB transmission dynamics. Nevertheless, *the TB problem is far from being solved, especially in developing countries*. There are *urgent needs* for control of the disease and thus it is essential to progress in *applied research*. Indeed, new vaccines [49,131], new drugs [23,134], and new diagnostics and advances in TB management [19,136] are urgently needed. Furthermore, we believe that it is no longer necessary to justify that *basic research*, including evolutionary and population genetics, experimental evolution, immunology, and cellular biology, is indispensable in order to progress in applied research. As we described above, molecular epidemiology is a scientific domain that can make the connection between applied and basic research. As demonstrated in this chapter, *molecular epidemiology studies may be useful in public health control and in management of clinical situations*. Nevertheless, today genotyping is exploited only in a few TB control programs and is usually done within the framework of retrospective studies. Very few studies have been conducted prospectively, and these mainly in developed countries [14,26,46,47,78,111,204]. Furthermore, it is essential first to extend these studies worldwide, particularly in developing countries. This requires, of course, financial and governmental support, an efficient and disinterested worldwide commitment, and technological improvement to develop molecular tools that are usable in developed as well as in developing countries. Second, the rapid exploitation of molecular data, *in real time*, is essential in order to control TB at global and local levels.

At the local level, it is crucial to rapidly and efficiently identify the source of contamination, the cases of cross-contamination, and the drug sensitivity of strains in order to select the best-adapted treatment and to rapidly propose prevention and treatment to patients' relatives when needed.

At a global level, the rapid international communication and global infectious disease surveillance and management are fundamental in order to identify an international outbreak

and prevent pandemia. This is relevant today not only for TB control but also for all the emerging infectious diseases such as avian flu in 2005, or Asian severe acute respiratory syndrome (SARS) in 2003, or mad cow disease in the 1990s. Constant concerted efforts at an international level spanning several decades can help solve the global TB problem, HIV/MTB co-infection, and drug resistance in both developed and developing countries. Global commitment and engagement of all interest groups will also be necessary to achieve this goal. To develop an efficient program of TB surveillance, interactive national and international databases are also required recovering all the epidemiological, molecular, and biological data of each isolate. As described in Section 1.3 of this chapter, a few databases already exist, but they require greater development (either they are not interactive or they are not updated regularly or they are too restricted). These databases should be accessible online and interactive in order to provide access to all clinicians and researchers so they can compare their data. They could be constructed on the model of nucleic acid or protein databases such as GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) or EBI (http://www.ebi.ac.uk/), which allow researchers to submit, consult, and analyze sequence data. To succeed, several conditions are needed: (i) the definition of standardized methodologies for data exploitation, (ii) the development of a completely disinterested structure that is updated frequently and that will avoid publication pressure and scientific competition, and (iii) the validation of data in order to prevent errors. This type of database may be a powerful public health tool to follow the evolution of TB from a drug resistance or epidemiological point of view at an international level. Several publications have shown the usefulness of databases: Drobniewski et al. [52], Filliol et al. [60], Zozio et al. [219], and Niobe-Eyangoh et al. [133].

1.5.2 Limitations of Modern Molecular Tools

Even today, three main caveats restrict the routine use of DNA fingerprinting: the cost, the complexity of the techniques, and the length of time needed to obtain results. Indeed, the high cost of most of these molecular techniques and the sophisticated equipment and skilled personnel required have precluded their implementation on a routine basis, especially in low-income countries. IS*6110*-based RFLP, spoligotyping, and automated MIRU-VNTR require sophisticated material and specifically trained personnel. Furthermore, globally the time between sputum collection and data interpretation is too long: (i) IS*6110*, spoligotyping, and classical drug resistance identification require mycobacteria culture lasting several weeks (see Section 1.2). In addition, only a limited number of strains can be rapidly identified at the same time for IS*6110*-based RFLP, spoligotyping, and MIRU-VNTR on agarose gel. However, MIRU-VNTR appears as the most appropriate technique to develop standardized data in a short time period with a larger number of samples. Indeed, automated MIRU-VNTR can analyze a high number of samples a day, which makes this technique promising in molecular epidemiology studies in real time, as it does not require cell culture (Supply, personal communication). Nevertheless, as described in Section 1.3 of this chapter, automated MIRU-VNTR requires sophisticated equipment and skilled personnel, and the cost is still high for low-resource countries. On the contrary, MIRU-VNTR on agarose gel is an easy technique with a lower cost, but only a few samples per day can be studied. Thus, at present, there are still no perfect molecular tools. In Chapter 41, Kathleen Victoir emphasizes that "*the creative scientific challenge at present is to develop the best possible tools adapted to resource-poor settings*." The perfect markers should be cheap, rapid, easy to use, and exportable between laboratories.

1.5.3 Promising New Technologies

The availability of whole genome sequences has aided the development of new genomic technologies such as *microarrays* or *genechips* (Fig 1.9). Today, this advanced technology is reserved for researchers in leading laboratories, but these DNA chips may soon invade hospitals and hopefully medical dispensaries. DNA microarrays are small, solid supports, typically glass, filter, or silicon wafer, upon which DNA molecules of known sequences are deposited or synthesized in a predetermined spatial order so that they can be made available as probes in a high-throughput, parallel manner. They can consist of a few hundred to hundreds of thousands of sets. There are three major applications for the DNA microarray technology: identifying the sequence (gene/gene mutation), determining the expression level (abundance) of the genes of one sample, or comparing gene transcription in two or more different cell types. Butcher [22] described the usefulness of microarrays for MTB research and their contribution for enhancing a TB control program. This review showed the broad application of microarrays in understanding MTB physiology, host–pathogen interactions, mechanisms of drug action, in vitro and in vivo gene expression, host responses, comparative genomics, and functional genomics of particular genes. As they can also help identify individuals with similar biological patterns, microarray analysis can assist drug companies in choosing the most appropriate candidates for clinical trials of new drugs. In the future, this emerging technology has the potential to help health care professionals select the most effective drugs, or those with the fewest side effects, for each patient. Butcher [22] stated that microarrays are one of the new functional genomics technologies exploiting genome sequence information that will bring us closer to reaching the scientific and moral imperatives of better vaccines, diagnostics, and new drugs for the control of TB throughout the world. They could help at all steps of the patient's follow-up: disease and strain identification, treatment selection, and observation of therapy efficacy. For the moment, cost is a limiting factor, but the objective of the specialists in biotechnology is to reduce the production cost in order to make this advanced technology routinely accessible.

Another method based on spoligotyping was recently developed by Cowan et al. [35]. The authors transferred

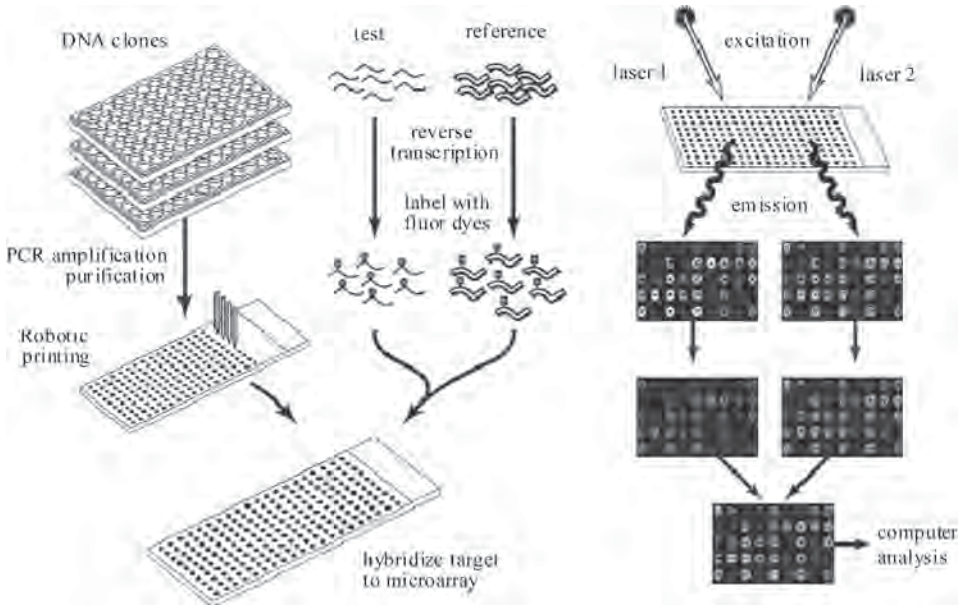


Fig. 1.9. cDNA microarray schema. Templates for genes of interest are obtained and amplified by PCR. Following purification and quality control, aliquots (~5 nl) are printed on coated glass microscope slides using a computer-controlled, high-speed robot. Total RNA from both the test and reference sample is fluorescently labeled using a single round of reverse transcription. The fluorescent targets are pooled and allowed to hybridize under stringent conditions to the clones on the array. Laser excitation of the incorporated targets yields an emission with a characteristic spectra, which is measured using a scanning confocal laser microscope. Monochrome images from the scanner are imported into software in which the images are pseudo-colored and merged. Information on the clones, including gene name, clone identifier, intensity values, intensity ratios, normalization constant, and confidence intervals is attached to each target (from [53], with permission).

spoligotyping from a reverse line-blot hybridization, membrane-based assay to a luminex multianalyte profiling system. This technique may offer many benefits such as a decrease in the turnaround time and the labor involved, a decrease in technical complexity, and greater flexibility (1–96 isolates can be used without increasing the labor time or cost per isolate, and reproducibility is increased) [35]. The authors demonstrate that the luminex system is an attractive alternative for laboratories that perform spoligotyping on a high-throughput scale or for those that frequently require a rapid turnaround time for only a few isolates per run [35]. Nevertheless, as a classical spoligotyping method, another technique with a greater discriminatory power would have to be used for obtaining a maximum of resolution.

Concerning genotypic susceptibility testing, the elucidation of the molecular mechanisms responsible for the action of various anti-tuberculous drugs facilitated the development of rapid methods for susceptibility testing. Jalava and Marttila [91] introduced genetic methods and new techniques useful for both resistance genetic studies and rapid molecular diagnostics of resistance for several bacteria including MTB. They described six different molecular techniques, from which two techniques held their attention: PCR single-strand conformation polymorphism (SSCP) and high-density oligonucleotide arrays. SSCP is a rapid screening method for base-pair alterations in PCR-amplified DNA. This method appears cost-effective and presents a short turnaround time, which makes it suitable for use in clinical laboratories. Jalava and Marttila [91] described that high-density oligonucleotide arrays may also offer a powerful solution to genotypic detection of drug-resistant MTB isolates. So far, these microarrays have mainly been used for the detection of rifampin resistance [72,123,198] with promising results. Consequently, Jalava and Marttila [91] argue that the DNA microarray strategy could be expanded to include parallel testing of various genes mediating drug resistance in MTB. Furthermore, an array for the simultaneous testing of isoniazid, rifampin, streptomycin, and fluoroquinolone susceptibilities has already been designed by Gingeras et al. [72] and could be integrated into a TB control program for the rapid diagnosis of drug-resistant TB. Jalava and Marttila [91] also defined the necessary requirements for assessing the suitability of molecular methods for anti-tuberculous susceptibility testing. First, the technique should have a high sensitivity because the amount of MTB cells in sputum varies and can be very low. Secondly, it should be able to detect minor drug-resistant subpopulations in a sample when the majority of the bacilli are susceptible. Two methods could help in this challenge: the invader assay [32] and on-chip ligase detection reaction [123]. The invader assay uses the thermostable flap endonuclease Cleavase VIII, derived from

Archaeoglobus fulgidus, which cleaves a structure formed by the hybridization of two overlapping oligonucleotide probes to a target nucleic acid strand [32]. This method can discriminate single-base differences. On-chip ligase detection reaction is applied to identify approximately 1% of mutant sequences in model samples consisting of mixtures of DNA from wild-type and resistant strains (see [123] for details). These technologies may be useful for clinical research in developed countries, but remain inaccessible for a TB control program on a large scale including low-resource countries.

1.6 CONCLUSION AND PERSPECTIVES

Despite the multitude of investigations launched in various scientific, clinical, and pharmaceutical domains on TB, this disease remains, along with AIDS and malaria, one of the three major killers among infectious diseases. In this chapter, we attempted to demonstrate (i) the contribution of molecular epidemiology in the understanding of transmission and pathogenesis of TB and (ii) the need for routine molecular epidemiology to improve TB surveillance and control programs at global and local levels.

As described by Smith et al. [166], it is clear that new approaches to preventing, diagnosing, and curing tuberculosis are needed, which depend on a better understanding of MTB and the host. They detailed that the National Heart, Lung, and Blood Institute developed recommendations for future TB research [166]. Among these different recommendations, all fundamental for fighting infectious diseases, five directly concern the domain of molecular epidemiology: (i) new resources for characterizing the MTB genome, proteome chips for more specific diagnoses; (ii) prospective studies associated with clinical trials in populations with TB or that are at risk for TB, to advance development of diagnostics and prognosis; (iii) genetic epidemiology studies; (iv) new quantitative and bioinformatics approaches to study the interaction between MTB and the infected host and how this influences the infection process; and (v) coordination between international organizations. This chapter provides evidence that all these points are of public health interest in the fight against TB. We believe that the fourth point is of particular importance, as it is now fully accepted in the scientific area of infectious diseases that the outcome of transmission, infection, and disease are dependent on both the intrinsic characteristics of the microbes and the host. Indeed, as developed by Hide et al. in Chapter 6 on leishmaniasis, integrated analysis of MTB genetics, MTB virulence factors, host immune responses, host genetics, as well as socioeconomic and environmental risk factors are all necessary for a better understanding of the interplay between these different factors and the risk of developing TB. This approach could also provide information on the critical biological pathways involved in the host resistance (latent infection) or susceptibility to TB and therefore help in orienting new therapeutic or vaccine strategies. Indeed, factors determining host resistant/susceptible status are complex and largely not clarified. Moreover, as demonstrated in this chapter, it has been suggested that the outcome of transmission and disease may be MTB strain dependent. This emphasizes the necessity of integrating different approaches to better understand the epidemiological situation's complexity.

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ABBREVIATIONS

AIDS:	Acquired immune deficiency syndrome
ATP:	Adenosine triphosphate
CAS:	Central Asian
cDNA:	Complementary deoxyribonucleic acid
DNA:	Deoxyribonucleic acid
DOTS:	Directly observed therapy short course
DR:	Direct repeat
DTU:	Discrete typing unit
EAI:	East African-Indian
FDA:	Food and Drug Administration
HIV:	Human immunodeficiency virus
IUATLD:	International Union Against Tuberculosis and Lung Disease
LAM:	Latin American and Mediterranean
LSP:	Large-sequence polymorphism
MDR-TB:	Multidrug-resistant tuberculosis
MIRU:	Mycobacterial interspersed repetitive units
MTB:	*Mycobacterium tuberculosis*
PCR:	Polymerase chain reaction
PPD:	Purified protein derivative
PTB:	Pulmonary tuberculosis
RLFP:	Restriction fragment length polymorphism
SNP:	Single nucleotide polymorphism
SSCP:	Single-strand conformation polymorphism
TB:	Tuberculosis
VNTR:	Variable number tandem repeat
WHO:	World Health Organization
ZN:	Ziehl–Neelsen

GLOSSARY

Allele: A variant of a single gene, inherited at a particular genetic locus; it is a particular sequence of nucleotides.

Allelic frequency: This index is the ratio of the number of a given allele to the total number of alleles in the population under survey.

Bacillus Calmette–Guérin (*BCG*) *vaccine*: A vaccine against tuberculosis that is prepared from a strain of the attenuated (weakened) live bovine tuberculosis bacillus, *Mycobacterium bovis*, that has lost its virulence by special culturing in artificial medium for years. The bacilli have retained sufficient antigenicity to become an effective vaccine for the prevention of human tuberculosis.

Bacteriophage: A virus that infects only bacteria.

Cell-mediated immunity: An immune response that does not involve antibodies but instead involves the activation of macrophages and natural killer cells, the production of antigen-specific cytotoxic T lymphocytes, and the release of various cytokines in response to an antigen.

Clone, clonal, clonality: From a genetic point of view, this term refers to all cases in which the daughter cells are genetically identical to the parental cell, whatever the actual mating system.

Cluster: Refers to a particular genotype shared by two or several MTB isolates.

Conjugation: Bacterial conjugation is the transfer of genetic material between bacteria through cell-to-cell contact.

Cost of resistance: Although mutations that provide resistance to an antibiotic can be considered beneficial, they often come with a physiological cost.

Endemic disease: Disease present or usually prevalent in a population or geographical area at all times.

Epidemiology: This scientific domain corresponds to the study of the distribution and determinants of health-related states and events in populations and the control of health problems.

Etiology: In medicine, the causes of diseases or pathologies.

Fitness: In biology, an individual's ability to propagate its genes.

Genetic drift: This phenomenon is a contributing factor in biological evolution in which traits that do not affect reproductive fitness change in a population over time. Although natural selection causes traits to become more prevalent when they contribute to fitness or eliminates those that harm it, genetic drift is a random process that affects traits that are more neutral.

Haploid: Refers to the ploidy level, that is, the number of copies of the basic number of chromosomes. Haploid cells bear one copy of each chromosome.

Hardy–Weinberg equilibrium: States that under certain conditions after one generation of random mating, the genotype frequencies at a single gene locus will become fixed at a particular equilibrium value. It also specifies that those equilibrium frequencies can be represented as a simple function of the allele frequencies. "Allele frequency" is a term from population genetics that is used in characterizing the genetic diversity of a species population, or equivalently the richness of its gene pool.

Housekeeping gene: A gene that codes for proteins needed all the time for agent survival and multiplication.

Immunosuppression: This immunological status occurs when T and/or B clones of lymphocytes are depleted in size or suppressed in their reactivity, expansion, or differentiation.

Linkage disequilibrium: The nonrandom association of alleles at two or more loci.

Locus: The position of a gene (or other significant sequence) on a chromosome. A locus can be occupied by any of the alleles.

Molecular clock: Refers here to the speed of evolution of a given molecular marker.

Natural selection: A process by which biological populations are altered over time, as a result of the propagation of heritable traits that affect the capacity of individual organisms to survive and reproduce. It is one of several mechanisms that give rise to the evolution of biological species (other mechanisms include genetic drift and gene flow).

Pandemic: Corresponds to a global epidemic and refers to an outbreak of an infectious disease that affects people or animals over an extensive geographical area.

Phagocytosis: This process involves the ingestion and digestion by phagocyte cells of microorganisms, insoluble particles, damaged or dead host cells, cell debris, or activated clotting factors. The principal phagocytes include the neutrophils and monocytes (types of white blood cells).

Phenotype: The observable characteristics of an organism, the expression of gene alleles (genotype) as an observable physical or biochemical trait. It is the result of interaction between the genotype and the environment.

Phylogeny: This scientific domain studies the evolutionary history of a species or group of related species.

Polymerase chain reaction (*PCR*): A technique used to amplify a specific region of DNA. An excess of two amplimers, oligonucleotide primers complementary to two sequences that flank the region to be amplified, are annealed to denatured DNA and subsequently elongated, usually by a heat-stable DNA polymerase from *Thermus aquaticus* (Taq polymerase).

Population genetics: This scientific domain studies the distribution of and change in allele frequencies.

Prevalence: The prevalence of a disease is defined as the ratio of the number of cases of a disease present in a population at

a given time and the number of individuals in the population at that time.

Random mating: This process involves the mating of individuals regardless of any physical, genetic, or social preference. In other words, the mating between two organisms is not influenced by any environmental, hereditary, or social interaction. Hence, potential mates have an equal chance of being selected.

Recombination: In molecular biology, "recombination" generally refers to the molecular process by which alleles at two genes in a linkage group can become separated. In this process, alleles are replaced by different alleles from the same genes, thereby preserving the structure of genes. One mechanism leading to recombination is chromosomal crossover.

Saprotroph: An organism that obtains its nutrients from nonliving organic matter, usually dead and decaying plant or animal matter, by absorbing soluble organic compounds. Because saprotrophs cannot make food for themselves, they are considered as a type of heterotroph (an organism that requires organic substrates to obtain its carbon for growth and development).

Segregation: In biology, this process refers to the separation of homologous chromosomes during mitosis and meiosis.

Symbiosis: An interaction between two organisms living together in more or less intimate association or even the merging of two dissimilar organisms.

Taxonomy: This science refers to the theory and practice of biological classification. This regroups the theories and techniques of naming, describing, and classifying organisms, the study of the relationships of taxa, including positional changes that do not involve changes in the names of taxa.

Transduction: The process in which bacterial DNA is moved from one bacterium to another by a bacterial virus (a bacteriophage, commonly called a phage).

Transformation: In bacteria, "transformation" refers to a genetic change brought about by taking up and recombining DNA, and "competence" refers to the state of being able to take up DNA.

Tuberculin skin test: Tuberculin (also called Mantoux test, currently named Purified Protein Derivative PPD) is an antigen used to aid in the diagnosis of tuberculosis infection. A standard dose of Tuberculin is injected intradermally (into the skin) and read 48–72 h later. A person who has been exposed to the bacteria is expected to mount an immune response in the skin containing the bacterial proteins.

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CHAPTER 2

Diseases that Threaten Livestock

J. Blancou[1] and P.-C. Lefévre[2]

[1]*Honorary Director General of the World Organisation for Animal Health (OIE), 11 rue Descombes, 75017 Paris, France*

[2]*Inspecteur Général de Santé Publique Vétérinaire, 127 rue d'Avron, 75020 Paris, France*

2.1 INTRODUCTION

At the end of fourth century, after the sanguinary eruption of the Huns under Attila and the expulsion of the Goths from Hungary, there was an exceptional famine and a deadly epidemic. A most severe and memorable epizootic began in the eastern part of Europe and spread westward. Curative measures proved useless, and "no healthy animal was safe unless it was branded on the forehead with a red-hot iron in the form of a cross," said Cardinal Baronius in *Annales Ecclesiasticae*, Vol. IV [8]. Severus Sanctus Endelechius, the Roman orator and poet, complained "And all that the work has produced, through all the period of life, was lost in two days, so rapid was the course of the misfortunes" [1].

At that time, epidemics of various kinds reigned throughout the world and caused incalculable mortality in human beings. George Fleming strongly suspects that, as in the fourth century, these epidemics were often accompanied by, if they did not precede and perhaps cause, directly or indirectly, those widespread diseases in mankind [8].

Throughout history, there are numerous records emphasizing the links established between human and animal mortality. Even today, deaths of humans and animals may be caused by the same agent or not, but are closely dependent on each other.

This chapter will thus aim at demonstrating the importance, for human health or wealth, of some selected animal diseases, listed in Table 2.1.

As there are so many of these diseases, this chapter will deal only with

- those that were very important in the past, but are now under control;
- those that are an economic burden and a serious obstacle to international trade; and
- finally, those that are transmissible to humans.

An explanation will then be given on how animal diseases can be controlled, or hopefully eradicated, thanks to modern technologies that are now available to veterinarians.

2.2 ANIMAL DISEASES UNDER CONTROL

2.2.1 General Considerations

Contrary to common opinion, the threat of animal diseases has been considerably reduced since the beginning of the twentieth century. Most of the plagues that were feared by the farmers or the veterinarians during the past centuries have been progressively brought under control.

For instance, when the World Organisation for Animal Health (OIE) was created in 1924, only nine diseases of livestock were listed that were considered as able to spread rapidly from one Member Country to another and thus put trade in animals and animal products at risk. These diseases were anthrax in all species, classical swine fever, dourine in horses, foot and mouth disease (FMD) in ruminants and pigs, glanders in equidae, contagious bovine pleuropneumonia, rabies in all species, and rinderpest in cattle and sheep/goat-pox. At a later stage, eight livestock diseases were added: African horse sickness, African swine fever, bluetongue in ruminants, lumpy skin disease in cattle, Rift Valley fever in ruminants, Teschen disease in swine, vesicular stomatitis in herbivores, and swine vesicular disease.

Since then, three of these diseases (dourine, glanders, and Teschen disease) have been considered as eradicated or restricted to such small areas that they can no longer threaten

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TABLE 2.1 Main Animal Diseases of Importance (Past or Present) for Animal Health, Animal Production or Trade, and Public Health

Animal disease	Pathogen – Main host	Present distribution	Transmission to humans	Control	Comments
African horse sickness	RNA Virus *(Orbivirus)* Equines	Mainly Africa and Arabic Peninsula	No	Vaccination	
African swine fever	DNA Virus *(Asfaviridae)* Swine	Mainly Africa. Eradicated from Spain and Portugal, DNA Virus still present in Italy (Sardinia) in Italy	No	Stamping-out	High importance in de loped countries
Anthrax	Bacterium *(Bacillus anthracis)* All mammals (birds in zoo)	World-wide distribution *	**Yes**	Vaccination	Importance in case of bioterrorism
Bovine spongiform encephalopathy	Prion Cattle	Mainly Europe	**Yes**	Stamping-out	Very high economic impact due to the cost of the control and eradication measures
Bluetongue	RNA Virus (Orbivirus) Sheep	World-wide distribution	**No**	Vaccination	Economically important in some countries (USA, SouthAfrica, etc.) – negligible in most African countries
Brucellosis	Bacterium *(Brucella spp.)* Herbivores	World-wide distribution	**Yes**	Vaccination or stamping-out	Important zoonotic aspect – transmission to humans by animal products (milk, cheese, etc.)
Classical swine fever (Hog cholera)	RNA Virus *(Pestivirus)* Swine	Central and South America – Asia – part of Europe	No	Vaccination or stamping-out	High economic impact in countries with a major pig industry
Contagious bovine pleuropneumonia	Bacterium *(Mycoplasma mycoides subsp. mycoides SC)* Cattle	Mainly Africa	No	Vaccination or stamping-out	
Foot and mouth disease	RNA Virus *(Aphtovirus)* *(Aphtovirus)* Cloven-hoofed animals	Mainly Africa and Asia	No	Vaccination or stamping-out	Very high economic significance in developed countries with a major dairy industry
Glanders	Bacterium *(Burkholderia mallei)* Equines: horses, donkeys, mules	Middle East, India, Asia, South America	**Yes**	Stamping-out	Rare and sporadic occurrence

Influenza	Virus *(Influenza spp.)* Mainly swine and Equines	World-wide distribution	**Yes**	Vaccination	Very high importance due to transmission to humans
Leptospirosis	Bacterium *(Leptospira interrogans)* All species	World-wide distribution	**Yes**	Vaccination	Usually low impact on animal productions, risk for human health
Lumpy skin disease	DNA Virus (Capripox) Cattle	Africa	No	Vaccination	Low impact – important only in countries with a major leather industry
Q fever	Bacterium *(Coxiella burnetii)* Ruminants	World-wide distribution	**Yes**	Hygiene and vaccination	Little economic significance
	Rabies RNA Virus *(Lyssavirus)* All mammals		**Yes**	Vaccination or stamping-out	Not a major animal disease (except in South America)
Rift Valley fever	RNA Virus *(Phlebovirus)* Ruminants	Africa and Arabic Peninsula	**Yes**	Vaccination	Economic significance due to increase of abortion rates – Important zoonotic aspect
Rinderpest	RNA Virus *(Morbillivirus)* All cloven-hoofed animals (mainly cattle)	Restricted to the Horn of Africa	No	Vaccination or stamping-out	RP should be eradicated soon at a global level
Sheep and goat pox	DNA Virus *(Capripox)* Sheep/goats	Africa, India, Asia	No	Vaccination	A major constraint in some developing countries
Tuberculosis	Bacterium *(Mycobacterium bovis)* Ruminants	Worldwide distribution	**Yes**	Stamping-out	High economic significance due to the control measures to protect humans
Vesicular diseases	Virus *(Vesiculovirus and enterovirus)* Cattle and swine	Europe/America	No	Vaccination	Importance due to the possible confusion with foot and mouth disease

* 'World-wide distribution' means that the disease is ubiquitous and can be introduced or reintroduced at any time in most of the countries having a large livestock industry. It does not mean that there is no country free from the disease at the present time: to obtain information on the current epidemiological situation of each country, kindly consult the Web site of the *World Organisation for Animal Health* (OIE) http://www.oie.int/fr.

international trade. In the meantime, four other diseases were reported in such a limited number of developing countries that there is hope for achieving their eradication or controlling efficiently their extension: African horse sickness, African swine fever and rinderpest [11,13] and, partly, contagious bovine pleuropneumonia.

The eradication or control of these serious diseases is due to the combined actions of farmers and veterinarians. Improving the general hygiene of animal husbandry played a key role in some cases (e.g., to control brucellosis and tuberculosis or to eradicate dourine and Teschen disease); however, it was the discovery of efficient veterinary vaccines that really paved the way for progressive control of the most contagious diseases (African horse sickness, contagious bovine pleuropneumonia, and rinderpest). At the same time, the progress achieved in diagnostic techniques and the application of these methods by the national veterinary services allowed them to recognize and destroy the animals infected or exposed to contagious diseases (e.g., glanders, almost eradicated using the mallein test). The same techniques are still used on a large scale to combat tuberculosis (using the tuberculin test) or other diseases that cannot be controlled by vaccination (using modern diagnostic techniques, see below). Should it be feared that some of these diseases could spread again in the future? The risk seems extremely low for two reasons:

- Firstly, an international concerted control is now in force at the world level through the World Organisation for Animal Health, and it has better chances of succeeding than when organized at the national level only, as it allows the reduction of prophylaxis costs, and specifically the improvement of the overall results, by avoiding the new contamination of one country by another.
- Secondly, it is clear that animal diseases do not present the same danger today as they did a century ago. The development of hygiene and asepsis followed by the discovery of vaccines, and later of sulfones and antibiotics, ended the ancestral scare of glanders, tuberculosis, or brucellosis, despite some recent fears from bioterrorism threats.
- For the same reasons as above, it is very likely that any new contagious disease of livestock that could emerge anywhere in the world would be immediately reported and contained with the help of national or international organizations. The emergence that is to be most feared today is the development of resistance to antibiotics by bacteria, which could become a serious obstacle to the control of some animal diseases and zoonoses.

2.2.2 Description of the Diseases

African horse sickness is an insect-transmitted disease caused by an Orbivirus of the family Reoviridae. It mainly affects equidae (horses, donkeys, zebras, etc.) and occasionally other species (such as dogs), but not humans (with the exception of a report of contamination in laboratory workers). It is transmitted by a biological vector, generally an insect of the genus *Culicoides*. The main signs are listlessness, hyperthermia, facial and thoracic oedema, and a frothy discharge. There are various clinical forms: subacute (cardiac) and acute (pulmonary), leading to death within several days, the mortality rate ranging from 10% in donkeys to over 90% in horses. The disease is enzootic in the central tropical regions of Africa, but has been known to make incursions into southern Europe and the Middle East. There is a specific preventive vaccination, but no known treatment.

Anthrax has an almost worldwide distribution due to the ease with which the spores of the causal agent can be disseminated in infected products, especially skins and bone meal. Anthrax is still a formidable zoonosis, which kills thousands of animals and many human beings every year. The causal agent, *Bacillus anthracis*, is a bacterium of the family Bacillacae, which forms spores that can survive in the soil for over 100 years. All mammals, including humans, are susceptible to the disease. Anthrax is most frequently transmitted by ingestion of spores. Infection, particularly in humans, can also occur by contact (through skin abrasions), insect bites, or inhalation of spores leading to a very dangerous form for humans. In ruminants, the most common forms are acute or peracute and death may occur in less than 24 h. In equids, digestive signs are more marked, and in swine, there is frequently inflammation and oedema of the pharyngeal lymph nodes. In carnivores, the pharyngeal or intestinal forms are the most common, with fulminant hemorrhagic septicemia. In humans, spontaneous recovery usually occurs in the cutaneous form ("malignant pustule"), whereas the intestinal and pulmonary forms are invariably fatal unless treatment is given. In the United States, from October 4 to November 2, 2001, 10 cases of bioterrorism-related inhalatory anthrax were registered in human beings, with 5 deaths.

To protect the animals from the disease, several measures can be recommended, such as avoiding contaminated land and food, burning or burying under quicklime-contaminated carcasses or material "litter," imposing quarantine. Vaccination of animals is possible and vaccines for humans are also available; however, not being very satisfactory, they are recommended only for those who are at risk due to their professional occupation. Penicillin is effective if administered early.

Glanders is a severe contagious equine disease, transmissible to humans. Due to the disappearance of large concentrations of horses, improvements in the conditions under which horses are kept and the discovery of "mallein," there are now only a few sporadic cases of glanders occurring in Africa and Asia. Nevertheless, due to the seriousness of the disease and the risk of transmission to humans, veterinarians and physicians continue to be extremely vigilant. The causal agent, *Burkholderia mallei*, can survive in the external environment for up to 6 weeks. Equids (donkeys in particular), more rarely goats and camels, and occasionally domestic or wild carnivores and humans (Fig. 2.1a) are susceptible. Transmission of glanders is indirect, by digestive or tegumentary route in equids. Humans become infected by handling contaminated objects and virulent material or during postmortem examinations of glanderous animals. The pathogen can be found in all tissues and excretions of animals. In the acute form, which

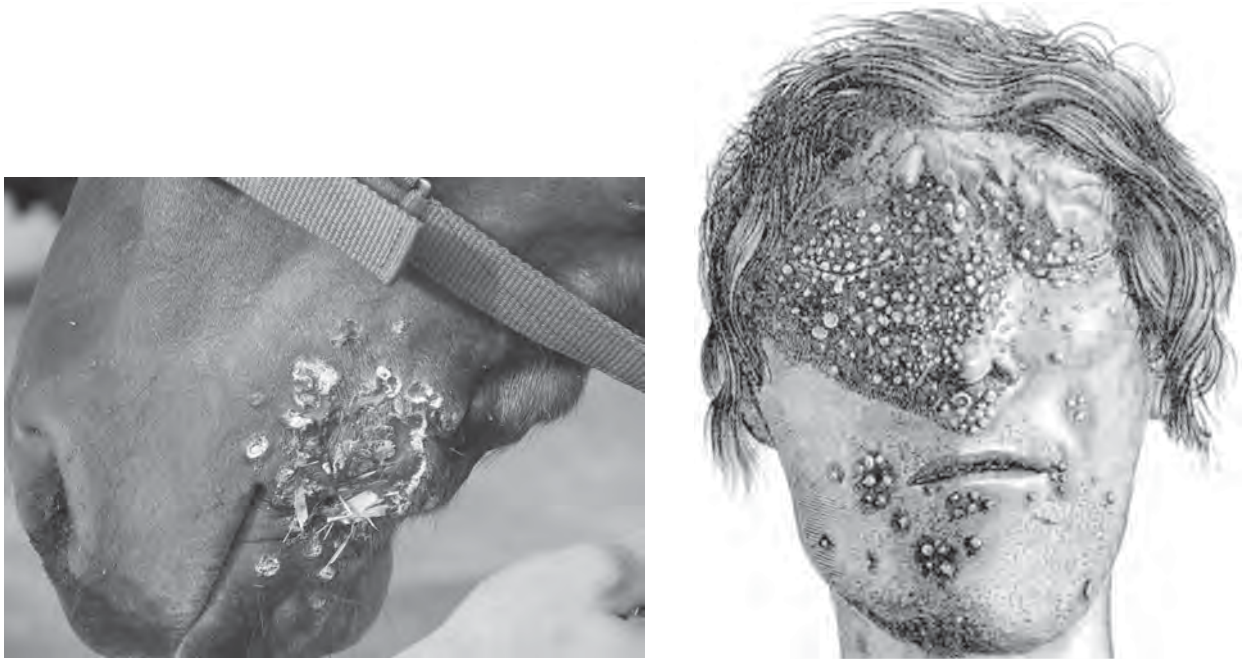

Fig. 2.1. (a) Glanderous horse (photo T.Morton / U.Wernery, 2004). See color plates. (b) Glanders in a veterinary student 1844, Musée Fragonard Alfort.

mainly occurs in donkeys, the disease is fatal within 1–4 weeks. It is characterized by high fever, followed by ulcers of the skin (Fig. 2.1b) and the nasal mucosae.

The cutaneous ulcers discharge an oily pus and lead to localized adenitis ("farcy buds"). The lymph vessels connecting the lesions (farcy cords) also become ulcerated. In chronic glanders, temperature fluctuates and death occurs only after several months. There may also be articular or genital complications. Sanitary prophylaxis is based on the testing of sick and contaminated animals by injection of "mallein" (equivalent to the tuberculin test for tuberculosis testing), with systematic elimination of positive reactors. Treatment by antibiotics is effective.

Rinderpest is a highly fatal contagious disease of cattle. The most spectacular livestock losses in the past were those caused by this disease: most authors estimate that 200 million cattle died from it in Western Europe during the eighteenth century, with 10,000 deaths per day between 1711 and 1769! [1]. From antiquity, this disease has been one of the most feared diseases of cattle in Africa, Asia, and Europe. It causes spectacularly high mortality and has extremely severe socioeconomic consequences. In the recent past, the disease has been gradually controlled through sanitary prophylaxis or vaccination, and it only persists in remote areas of Somalia. If the Global Eradication Programme is still carried out, rinderpest may become the first animal disease to be eradicated at the world level. The causal agent is a virus of the family Paramyxoviridae, genus *Morbillivirus*, which can remain viable for long periods in chilled or frozen tissues [11]. The disease can be transmitted by direct or close indirect contact. Ruminants and swine, domestic or wild, are susceptible to the disease, which is not transmissible to humans. Blood and all tissues are infectious before the onset of the clinical signs, and infection is via the epithelium of the respiratory tract. In the classic form observed in cattle, the disease begins with a febrile period, with anorexia and dyspnoea. This is followed by mucous membrane congestion, with erosion of the oral mucosae (Fig. 2.2).

Gastro-intestinal signs appear several days later, when the fever subsides: profuse hemorrhagic diarrhea, decumbency, and death. In sheep, goats, and pigs, pyrexia, anorexia, and diarrhea may also be observed. Sanitary prophylaxis is based on the isolation or slaughtering of sick and in-contact animals, destruction of cadavers, disinfection and protection of free zones. The commonly used vaccine is an attenuated strain of rinderpest virus. Immunity lasts at least 5 years and is probably life long. There is no treatment for rinderpest.

Fig. 2.2. Rinderpest in an African calf (FAO/OIE document). See color plates.

Teschen disease is a particularly virulent, highly fatal disease of pigs. It is caused by strains of porcine enterovirus serotype 1 (PEV-1) of the genus Teschovirus. The disease was first described in Czechoslovakia in 1929. During the 1940s and 1950s, it caused serious losses in European countries and spread to Madagascar in 1950, where 200,000 pigs died in less than 5 years. The clinical disease is now rare, although serological evidence indicates that virus variants, which are not pathogenic, still circulate in pig populations.

2.3 DISEASES THAT ARE AN ECONOMIC BURDEN AND HAMPER INTERNATIONAL TRADE IN ANIMALS AND ANIMAL PRODUCTS

2.3.1 General Considerations

Several transmissible animal diseases are still prevalent in some regions of the world where they represent an important economic burden for the countries affected. All these diseases also represent a permanent threat for other countries, which are free from the disease and wish to import animals or animal products from the infected regions. The World Organisation for Animal Health thus updates, on a yearly basis, the *Terrestrial Animal Health Code*, which recommends the measures necessary to avoid such incidents [14]. The assessment of the actual economic impact of a disease nevertheless remains difficult, for certain components are imprecise or even subjective, in particular when statistics from one country or epoch are compared with those from another country or epoch. There was a time when an epidemic of African horse sickness would be much more serious for the national economy and even for the security of a country than an epidemic of FMD. In addition, there are countries where nowadays any disease of any domestic species means poverty, famine, and, at times, human deaths. To attempt an economic evaluation of incidents related directly to the importation of a disease, the authors have used the livestock unit (cattle, pigs, equines, etc.) or, in the case of recent epidemics, estimated losses are converted to US$. The available data are shown in Table 2.2.

Below are some examples of the history of the extension of these diseases and of their cost for those countries where they were introduced. The best documented examples of the spread of some so-called "transboundary diseases" are the following:

- *African swine fever* is enzootic in a large part of tropical Africa. From this region, it was exported to Algeria in 1939 (purchase of live pigs) and to Senegal in 1959 (purchase of pig meat). It was no doubt pig meat, infected with African swine fever virus and present in kitchen waste unloaded from ships or aircraft, that infected parts of Europe in 1957 (Portugal, followed by Spain and France), and from there to Cuba in 1971–1980, then Haiti, the Dominican Republic, Malta and Brazil in 1978/1979 [2].

TABLE 2.2 Examples of Economic Losses Following the Introduction of Diseases of Animals into Countries or Regions*

Imported animals or animal products	Disease introduced	Losses** following introduction Country of origin/country affected (dates)
Cattle or products of bovine origin	Rinderpest	Europe: 200 million (18th century) Central Europe/Netherlands: 875,000 cattle (1766) Estonia/UK: 500,000 cattle (1865) Somalia/Southern Africa: 5.3 million cattle (1898) East Africa/West Africa: 500,000 cattle (1914)
	Bovine spongiform encephalopathy	UK/Ireland: US$ 1.3 million (1990/94) Argentina/UK: 430,000 susceptible animals (1967/68)
	Foot and mouth disease	Brazil/Mexico: US$ 150 million (1946) Europe/Denmark: US$ 1.6 million (1982) Middle East/Itlay: 11,897 susceptible animals (1993)
	Contagious bovine pleuropneumonia Theileriosis	Netherlands/UK: 200,000 cattle (1869) Mozambique/South Africa: 900,000 cattle (1904) Bosnia and Serbia/Hungary: 640,000 pigs (1896)
Pigs or pig products	Classical swine fever	Europe/belgium: 1 million pigs (1990/91) Europe/Germany: 1.5 million pigs, US$ 1 billion (1993/1994)
	African swine fever	Africa/Cuba: 536,000 pigs (1971) East Africa/South Africa: 70,000 horses (1854) Middle East/Asia: 170,m000 horses (1959)
Equines	African horse sickness	Africa/Middle East: 300,000 horses (1969) Spain: 2,000 horses (1989)

*Adapted and updated from J. Blancou and F. Meslin [2].
**Losses may arise from the direct effects of the disease, or from the control measures adopted to control it (mostly through a slaughter policy).

- *Classical swine fever* was first identified with certainty in Ohio in 1830, and its rapid spread was responsible for losses amounting to $100 million in the United States. From this country, it spread widely throughout the world, only a few regions escaping. Great Britain became infected in 1860, followed by Sweden and Denmark (through breeding stock). In Hungary, in 1896, 860,000 pigs developed the disease and 640,000 died [2].
- *Foot and mouth disease (FMD)* began its extension across Europe as early as 1546. At the beginning of the twentieth century, it was exported with European cattle to South America, from whence it returned much later (1967) to England with meat products. The disease also reached Mexico with Brazilian cattle, causing a very severe epizootic between 1946 and 1954 [2]. Between 1977 and 1987, a total of 1923 outbreaks of FMD occurred in 12 European Community Member States, with an average cost of US$ 160,000 per outbreak [5]. Nevertheless, this cost was estimated to be lower than the cost of annual vaccination against the disease; in 1991, vaccination was, therefore, prohibited in the European Community. However, in 2001, a very severe epizootic struck the United Kingdom: nearly 6 million heads (essentially sheep and cattle) had to be sacrificed to get rid of the disease. Total economic losses amounted to 2700 million Euros, and it is now debated in the European Union whether vaccines should be used on a local basis in case of new outbreaks.
- *Sheep-pox* is generally confined to the arid zones of Africa and Asia. Nevertheless, France became infected by sheep purchased from North Africa; Great Britain by Merinos purchased from Spain (1846) and later by Dutch sheep (1947); Annam by a ewe purchased in Hong Kong, and so on. Total losses have been heavy: in France, sheep-pox was responsible for the death of over a million sheep in the eighteenth century. These losses have been considerably reduced after the discovery of safe and efficient vaccine.
- *Bovine spongiform encephalopathy (BSE)* is the best example of a recent episode of a trade-related disease. On several occasions, the United Kingdom exported cattle affected by BSE and contaminated meat meal to numerous countries. Many cattle died from the disease, or were sacrificed to protect public health, in Canada, France, Germany, Ireland, Portugal, Switzerland, and so on. In less than a year, the cattle producers in Canada lost more than US$ 5 billion, after the notification of a single case of BSE in this country in May 2003.

2.3.2 Description of the Diseases

African swine fever is a contagious disease of domestic and wild swine caused by a virus of the family Asfarviridae (but was formerly classified in the family Iridoviridae). It is transmitted by direct or indirect contact (contaminated feed) or by ticks of the genus Ornithodoros ("soft ticks"). It causes high fever, diarrhea, ataxia, and a congestion of the skin. There are acute, subacute, and chronic forms. Mortality is very high in domestic pigs, whereas the disease often remains unapparent in wild African swine, which may act as a reservoir for the virus. The disease is enzootic in Africa, but in the past, there have been incursions into other regions (Latin America, Europe). There is no treatment or vaccine.

Aujeszky's disease is a contagious disease principally affecting swine, which constitute the principal host and reservoir of the causal agent, a *herpesvirus*. The main clinical signs of the disease in pigs are fever, anorexia, respiratory, intestinal and neurological signs and abortion. Sporadic cases also occur in other species (ruminants, carnivores). In such cases, the disease progresses rapidly toward death, the animal presenting neurological signs and often very violent pruritis (Aujeszky's disease is also called "mad itch"). Aujeszky's disease occurs in most countries. There is no treatment, but vaccination is possible.

Bluetongue is a noncontagious disease of ruminants caused by an *Orbivirus* (24 serotypes are known) generally transmitted by insects of the genus *Culicoides*. Only sheep express clinical signs of the disease: fever, erosions of the oral mucosae, cyanosis of the tongue, lameness, abortion, pneumopathy. Stillbirth is high. Other species present only a serological reaction to infection, especially cattle that act as a reservoir of the virus. The disease is widespread in hot countries, but is rarely clinically expressed. In the last 10 years, the geographic distribution of the vector *Culicoides imicola* and of the disease has extended to Mediterranean countries (Italy, Spain, and France), probably due to "global warming" then to northern Europe in 2006 with *Culicoides dewulfi* as a new vector. There is no treatment, but specific vaccines exist.

Classical swine fever is a contagious disease of domestic and wild swine, caused by a Pestivirus, and transmitted by direct or indirect contact (notably through contaminated feed). The acute form includes pyrexia, gastrointestinal problems (vomiting, diarrhea), respiratory (nasal discharge), or neurological (motor incoordination) signs and cutaneous lesions (erythema, cyanosis). Death occurs in about 12 days, though sometimes much later in subacute or chronic forms of the disease. There are also congenital forms of the disease. Classical swine fever is widespread in Asia, Africa, and parts of Latin America and Europe. There is no treatment, but there are effective vaccines.

Contagious bovine pleuropneumonia (CBPP) is widespread in Africa. It is also present in other regions of the world, including Southern Europe, the Middle East, and parts of Asia. The causal agent, *Mycoplasma mycoides* subspecies *mycoides* SC (bovine biotype) is deprived of cell walls and is therefore pleomorphic and resistant to antibiotics of the β-lactamine group, such as penicillin. The germ is not very resistant in the environment. Bovines are the main victims of the disease. Transmission occurs mostly by direct contact (coughing, saliva, urine). Unapparent carriers are a major source of infection. In adult animals, the main symptoms are moderate pyrexia with respiratory, pulmonary, and pleuretic symptoms. The disease can be peracute (very rapid death), subacute, or chronic (Fig. 2.3).

In young animals, pulmonary tropism is not the general rule and infected calves may present arthritis. Postmortem examination reveals a large amount of yellow or turbid exudate in the

Fig. 2.3. Contagious bovine pleuropneumonia in an African cow (FAO document).

Fig. 2.4. Foot and mouth disease: lesions on the tongue (photo J.F. Valarcher, 2006). See color plates.

pleural cavity (up to 30 L) that coagulates to form large fibrinous clots. Sanitary prophylaxis in disease-free areas is based on quarantine, serological tests and slaughtering of all animals with specific antibodies. Control of cattle movements is the most effective way of limiting the spread of CBPP in infected countries, where a CBPP vaccine can also be used if allowed by national or regional regulations. There is no really effective treatment, and antibiotic treatment is not recommended, to avoid any antibioresistance and the persistence of the disease.

Equine influenza is an acute, contagious respiratory disease caused by two distinct subtypes (H7N7, formerly equi-1, and H3N8, formerly equi-2) of influenza A viruses within the genus *Influenzavirus* A of the family Orthomyxoviridae. In fully susceptible animals, clinical signs include pyrexia and a harsh dry cough followed by a mucopurulent nasal discharge. Characteristically, influenza spreads rapidly in a susceptible population. There is no treatment for the disease, but spread of infection and severity of disease may be reduced by the use of potent inactivated equine influenza vaccines containing epidemiologically relevant virus strains.

FMD is one of the most highly contagious animal diseases, capable of causing serious direct or indirect losses. Mortality is low in adults, but often high in young animals, with deaths occurring due to myocarditis. FMD is enzootic in certain parts of Asia, Africa, the Middle East and the north of South America. The causal agent is a virus of the Picornaviridae family, genus *Aphtovirus*, which can remain virulent up to 1 month in meat or in the environment. Seven serotypes are known that can cause different epizootics, as there is no complete cross protection between these serotypes. Cattle, sheep, goats, pigs, domestic or wild, are the most susceptible species; human beings are not receptive. The virus can be transmitted by direct or indirect contact. The main sources of the pathogen are sick animals during the incubation period. The main symptoms are pyrexia, anorexia, shivering, reduction in milk production, drooling, lameness caused by the vesicles (aphtae), which develop on buccal (lingual) and nasal mucous membranes (Fig. 2.4) and/or on the udder or between the claws.

Complications may occur, including abortion, or death of young animals. In pigs, severe podal lesions may occur, but in sheep and goats, foot lesions may go unrecognized. Protection of FMD-free zones is achieved by control of animal movements, slaughter and destruction of exposed animals, destruction of virulent matters, and quarantine measures. When the above measures are not acceptable by the officials, the farmers or the public, some or all herds may be vaccinated. There is no treatment for FMD.

Lumpy skin disease is a contagious disease of bovines caused by a *Capripoxvirus* transmitted solely by mosquitoes or biting flies. The disease is not always clinically expressed: it is characterized by fever, oculonasal discharge, lameness, and nodules at the level of the mucous membranes or skin on all parts of the body, with a lymph node reaction. There is no treatment, but effective vaccines exist.

Peste des petits ruminants is a contagious disease of sheep, and especially goats, caused by a *Morbillivirus* antigenically similar to the rinderpest virus [12]. The disease is transmissible by direct contact. It is characterized by severe pyrexia, diarrhea, nasal discharge, bronchial pneumonia, and abortions. Cattle and pigs develop unapparent forms. Peste des petits ruminants occurs in Africa, Asia, and the Middle East. There is no known treatment, but it can be prevented using a homologous or heterogonous vaccine (rinderpest vaccine).

Sheep-pox and goat-pox are serious viral diseases currently circulating enzootically in most of Africa, the Middle East, and Asia, with occasional incursions into Eastern Europe. The causal agent is a virus of the Poxviridae family, *Capripoxvirus* genus that can survive for many years in dried infected material. It is transmitted through direct or indirect contact with contaminated implements and through insect bites. The characteristic cutaneous eruption on the body or in the oral cavity begins with erythematous areas, followed by papules (Fig. 2.5).

Nodules develop in the lungs, causing bronchopneumonia with coughing, abundant nasal discharge, depression, anorexia, and emaciation. Animals may recover in 20–30 days.

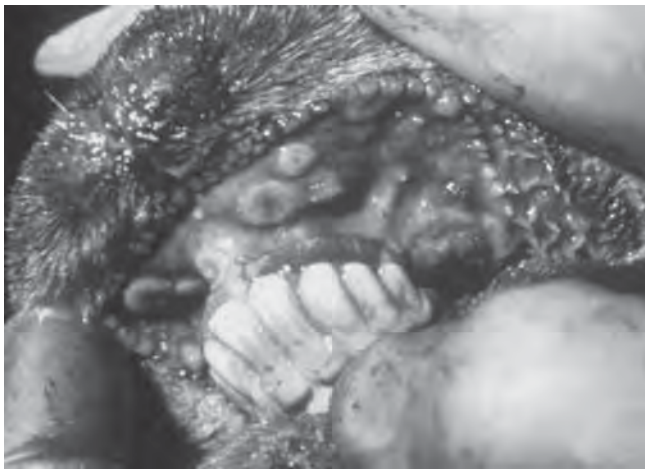

Fig. 2.5. Sheep and goat pox: mouth lesions (FAO document).

However, death is frequent when complications occur (e.g., abortion or pneumonia). The morbidity rate is 70–90% in enzootic areas, with a mortality rate of 5–10%. Sanitary prophylaxis is based on isolation of infected and sick animals for at least 45 days after recovery. Infected animals are sacrificed, infected cadavers and contaminated products are destroyed. Disinfection and quarantine as well as animal and vehicle movement controls are also necessary. There are numerous attenuated virus vaccines that can protect the animals up to 2 years. There is no treatment for sheep-pox.

Transmissible spongiform encephalopathies (TSEs) represent a group of neurodegenerative diseases characterized by a very long incubation period. The lesions are mostly restricted to the central nervous system. The outcome is always fatal, and, to date, there is neither cure nor medical prophylaxis for these diseases. They are recognized in several animal species (e.g., transmissible mink encephalopathy and chronic wasting disease of American deer) as well as in human beings (e.g., Kuru and CJD; see below). The causative agents of TSEs are usually called prions, as they are thought to be primarily composed of a pathological form of a host protein, the prion protein (PrP), the molecular and biochemical nature of which remain unclear. The most important animal TSEs from an economic point of view are scrapie in sheep and BSE in cattle.

Scrapie is a naturally occurring infectious neurodegenerative disease of sheep and goats characterized by vacuolar or spongy changes in the central nervous system. It has been recognized for over two and a half centuries and is now endemic in many parts of the world. The infection in sheep may be passed from ewe to lamb in the period from parturition to weaning. The majority of cases occur in sheep between 2 and 5 years of age. The disease is recognized by the clinical signs, which start insidiously with behavioral abnormalities, pruritus, and incoordination, and it is inevitably fatal. Prophylaxis is based on partial or complete depopulation of affected animals or herds and on the use of selective breeding programs based on PrP genotypes.

BSE, also known as "mad cow disease," was first recognized as an epizootic in the United Kingdom in 1987. It can be explained by oral exposure to a scrapie-like agent in the ruminant-derived protein of meat-and-bone meal included in proprietary concentrates or feed supplements. BSE has a peak incidence in cattle aged between 4 and 5 years. The clinical course is variable, but can extend to several months. Experimental transmissibility of BSE to cattle has been demonstrated following parenteral and oral exposure to brain tissue from affected cattle. Control measures are based on the partial or total depopulation of affected herds. As a result of these measures, the epizootics in most of the affected countries are on a decline, the peak of the epizootic having been reached in 1992 with more than 30,000 cases in the United Kingdom. Cases of BSE currently occur throughout most of Europe and have now also been detected in Asia and North America. There is evidence of a causal link between the BSE agent and a new variant form of the human TSE, Creutzfeldt–Jakob disease (CJD) in human beings.

Vesicular diseases other than FMD are important because of the similarity of their clinical signs with those of the most feared FMD.

Vesicular stomatitis is an infectious disease caused by a *Vesiculovirus* that can affect herbivores (especially horses), swine, and humans. The virus is transmitted by the transcutaneous route or by arthropod bites. Mortality due to the disease is low, but morbidity can reach 90%. It is characterized by hyper salivation and eruptions in the mouth, on the teats and on the claws, leading to possible confusion with FMD. It chiefly occurs in America. No treatment or vaccine is currently applied.

Swine vesicular disease is a contagious disease of pigs, caused by an *Enterovirus* and characterized by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout, and teats. Both diseases are extremely important for international trade in animals, as they are clinically indistinguishable from FMD and any outbreaks in pigs must be assumed to be FMD until investigated by laboratory tests.

2.4 ANIMAL DISEASES THAT MAY THREATEN HUMAN HEALTH

Humans can be affected by over 1400 different pathogens, the majority of which are zoonotic [3]. Many causes are favorable to the multiplication and diffusion of these zoonotic agents, that is, agents transmissible from animals to humans, and vice versa.

The most frequently reported causes are as follows:

- Animal and human diet changes: The number of human food-borne infections due to the ingestion of animal pathogens has considerably increased with the development of large-scale industrial food processing and the development of fast-food restaurants [17].

- Animal over-population, associated with modern breeding methods for domestic animals or overprotection of certain wild species.
- Human or animal population displacement, voluntary or not, notably following socioeconomic disorder or translocation. Similarly, increased human and livestock populations in Africa have led to major health problems. In recent years, the increase in livestock populations has slowed down due to a cycle of degradation and diseases, affecting especially traditional pastoral systems with a close physical association between people, livestock, and wild animals.
- Increased contact with a wildlife reservoir, associated with the development of hobbies (hunting, fishing, tourism, and especially "ecotourism"), which gives humans the occasion to be in contact with zoonotic agents excreted by healthy carrier animals [4] or with arthropods vectors of such agents, such as *Coxiella burnetii*, the agent of Q fever. This may also be the case with the bovine tuberculosis bacillus, which is spreading to new wildlife reservoirs, including wild carnivores, deer, or wild boars. Expansion of ecotourism-based industries, changes in land-use practices, and escalating competition for resources have increased contact between free-ranging wildlife and humans.
- Accelerated degradation of the natural environment, notably in developed countries (by deforestation, building of dams, land consolidation) may cause wildlife species to move to new areas, favoring their relocation in suburban zones, therefore entering into contact with humans [10]. The same type of risk can exist on farms, where the coexistence of different animal species can facilitate the development of several diseases. Global warming caused by human activities is also a cause of concern in the emergence of viral and bacterial vector-borne diseases, such as Rift Valley fever [13].
- The emergence of bacterial strains resistant to antibiotics and their widespread distribution, following an excessive usage or misuse in both human and veterinary medicines. This is notably one of the explanations for the emergence or reemergence of bacterial or viral food-borne pathogens.
- Finally, zoonotic agents, sought for by "bioterrorists," as they can simultaneously adversely affect human and animal health, could be spread voluntarily.

In some countries, the reemergence of bacterial zoonoses may be due to a lack of surveillance or a lack of appropriate control measures associated with the breakdown of public services [10]. This is caused more often by a lack of financial and human resources, which may be the consequence of economic crisis, social uprising, wars or natural disasters. The reemergence of some zoonoses, especially water- or food-borne zoonoses, is very often associated with an influx of refugees or insalubrities of poor districts in which the sanitary services can no longer exercise control.

Another factor in the emergence or reemergence of bacterial zoonoses is the impoverishment of some human populations among which all zoonoses can find suitable hosts due to poor hygiene.

- Paradoxically, in other cases, the risks are associated with the financial ease of upper social classes, which favors easier practice of hobbies: tourism, hunting, fishing, and so on. By practicing these hobbies, people may be exposed to potentially infected wild animals.
- For food-borne infections, the most unfavorable factor is the integration and globalization of food treatment chains, which multiply the risk of contaminations in an exponential way [18].

2.4.1 Description of the Diseases

Brucellosis is a contagious disease affecting numerous animal species and humans, caused by bacteria of the genus *Brucella*. This genus is divided into seven species that are generally host specific: *B. abortus* (bovids), *B. melitensis* (small ruminants), *B. ovis* (sheep), *B. suis* (swine), *B. canis* (canidae), and so on. The infection mainly involves the reproductive system (abortions, metritis, orchitis) and the locomotor system (arthritis). The disease can be treated by antibiotics (although treatment is not recommended in animals to avoid interference with eradication programs) and prevented by specific vaccines.

Leptospirosis is caused by infection with various serovar of the bacteria *Leptospira interrogans*. The disease affects almost all species (equids, swine, ruminants, carnivores, rodents, etc.), including humans. It can be asymptomatic or expressed as fever, jaundice, hemoglobinuria, and abortions. The pathogens are localized in the urogenital system and excreted in the urine. Transmission is usually caused by the skin or mucous membranes that come into contact with water or other material contaminated with urine. Antibiotic treatment can cure or prevent the disease, and there are specific vaccines.

Q fever (Query fever) is a zoonosis that occurs in most countries. Q fever is a highly infectious disease, which is caused by the proliferation of *Coxiella burnetii*, a small and pleomorphic bacterium. As an obligate intracellular bacterium, this agent can be grown only in embryonated eggs or cell cultures. The signs of Q fever in cattle include abortion, dead or weak offspring, retained placenta, metritis, and infertility. In small ruminants, Q fever is often associated with sporadic abortions or outbreaks of abortions followed by recovery without complications. *Coxiella burnetii* infection persists for several years, and is probably life long. Sheep, goats, and cows are mainly asymptomatic carriers, but can shed massive numbers of bacteria at parturition, and intermittently in various secretions and excreta. Other domestic animals, such as cats, rabbits, birds, and so on, are also susceptible to infection and should be considered as possible sources of infection for both animals and humans. In humans, Q fever occurs in either an acute form (self-limiting febrile episode, pneumonia, hepatitis) or a severe chronic form (endocarditis) following an early infection that may be passed unnoticed. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In some

countries, a vaccine is available for professionally exposed individuals.

Rabies is the most feared and widespread disease throughout the world, with the exception of a few countries. It causes relatively few deaths in domestic animals, and direct economic losses are therefore slight. However, its transmissibility to humans imposes costly preventive measures. In wild animals, mortality is often high. Rabies is caused by a *Lyssavirus* of the family Rhabdoviridae, genus subdivided into four serotypes and seven genotypes. Within a given serotype, there are variants and biotypes adapted to different hosts (dog, fox, bat, etc.). All warm-blooded animals are susceptible to experimental inoculation. Natural infection does not exist in birds, however. The susceptibility of mammals to natural infection depends on the virus strain, and once the disease has started, it is almost invariably fatal. Rabies is usually transmitted by intramuscular inoculation of virulent saliva (e.g., through bites). Saliva may be infectious up to 4 weeks before the onset of symptoms. The symptoms usually involve a change in behavior, and then various disorders: motor incoordination, paralysis, aggressiveness, and so on (Fig. 2.6).

Rabies can sometimes lead to death without any particular clinical signs, especially in bats. There are no macroscopic lesions characteristic of rabies, and the laboratory diagnostic is, therefore, of outmost importance. Isolation and/or slaughter of sick animals, wearing of a muzzles and quarantine are the measures usually recommended to protect rabies-free areas. In infected territories, preventive vaccines are usually administered to domestic animals at risk, by parenteral route. The oral route is also possible, especially in the case of wild animals. Postexposure vaccination is usually reserved for humans. There is no effective treatment for rabies after the onset of symptoms.

Fig. 2.6. Dog rabies: furious form (Photograph by Thiaucourt, Algeria, 1982).

Rift Valley fever is a major zoonosis caused by a Phlebovirus transmitted by many species of mosquitoes and characterized by a short incubation period. In young animals, the disease is usually fatal with fever, diarrhea, purulent discharge. In adults, especially cattle, sheep, and goats, the main clinical sign is abortion or a raised stillbirth level. In most of the cases, human beings present a mild form with hyperthermia and headaches, but in 5% of the cases, infection may lead to a very serious and fatal hemorrhagic fever. There is no treatment, but an effective vaccine exists.

Swine influenza is a highly contagious viral infection of pigs. Swine influenza virus infections cause respiratory disease characterized by coughing, sneezing, nasal discharge, elevated rectal temperatures, lethargy, difficulty in breathing, and depressed appetite. In some instances, infections are associated with reproductive disorders, such as abortion. Clinical signs and nasal shedding of virus can occur within 24 h of infection. Morbidity rates can reach 100%, whereas mortality rates are generally low. Secondary bacterial infections can exacerbate the clinical signs following infection. Transmission is through contact with virus-containing secretions, such as aerosols created by coughing or sneezing, and nasal discharges, and human beings may occasionally shed the virus.

Tuberculosis is an ubiquitous, usually chronic zoonosis, normally found in the respiratory tract and affecting almost all vertebrate species. The causal agent is a bacterium in the family Mycobacteriacae, genus *Mycobacterium*, and of various species: *M. tuberculosis* mainly found in humans, *M. bovis* mainly in cattle, and *M. avium* mainly in birds. It can survive in the external environment for several months. The disease is transmissible by the respiratory route (aerosols) in over 90% of cases. It can occur by ingestion of bacilli contained in the milk. The disease chiefly affects the respiratory system (coughing, dyspnoea, etc.), though it can also affect the digestive system (diarrhea, constipation, etc.) and other organs. Death is usually due to cachexia. Sanitary prophylaxis is based on the slaughter of animals reacting to the tuberculin test, disinfection of contaminated premises and implements, quarantine, pasteurization of milk, and so on. BCG vaccination in cattle was abandoned in the 1950s. It is now being reconsidered for use in extensive rearing systems and in some wildlife species. Treatment of animals with antibiotics or isoniazid is possible, but not recommended, to avoid the selection of resistant strains of *Mycobacterium*, which could thereafter infect human beings.

2.5 SURVEILLANCE AND CONTROL OF TRANSMISSIBLE ANIMAL DISEASES: PROGRESS EXPECTED FROM MODERN TECHNOLOGIES

It is well established that 75% of all emerging diseases, which have affected people over the last two decades, have occurred as a result of an animal pathogen moving into the human host [3]. The surveillance of animal diseases at the regional or

world level is, therefore, of utmost importance, for safeguarding both animal and human health.

This surveillance is based on the analysis and synthesis of information collected by official public health or animal health systems in each country. Data may be provided to health authorities through partnership and networks organized with the help of medical practitioners, veterinarians, animal health and wildlife specialists, or livestock breeders.

In some countries, this traditional surveillance system has been completed by the development of some more sophisticated systems using the remote sensing satellite warning systems, which collect the difference in atmosphere pressure, the sea surface temperature, or the differential vegetation index. Such techniques may alert authorities to the presence of virus/emergence well in time to enable them to take appropriate control measures, as was the case in Mauritania during the last outbreak of Rift Valley fever [9].

Furthermore, some other countries have taken advantage of the progress of computer technologies and have developed modeling systems, which allow better surveillance and prevision of the evolution of epizootics.

Current methods used to control animal diseases are mainly aimed at reducing its spread or at eradication through the two classical methods of sanitary or medical prophylaxis:

- Sanitary prophylaxis, which consists in slaughtering and/or destroying all infected or contaminated animals (stamping-out method), has largely been proven useful for the control of glanders, dourine, bovine tuberculosis, FMD, swine fevers, and so on.

In the specific case of FMD, the European Community carried out a risk assessment at the end of the 1980s to determine the best option between routine vaccination each year against the disease and nonvaccination (the stamping-out policy being applied in case of an outbreak). The outcome of this risk assessment predicted that a stamping-out policy would cost less than a vaccination policy, whatever the different scenarios used for the analysis [5]. However, this method of sanitary prophylaxis reaches its own limits when wild animal reservoirs are concerned, or when the public does not accept the destruction of too many animals. The stamping-out policy is also difficult to apply when a disease has spread throughout the world: all hope of one day eliminating the animal reservoirs of anthrax, tularemia, leptospirosis, or any other ubiquitous disease seems vain.

- Medical prophylaxis, which is based on either parenteral vaccination of animals or chemoprophylaxis, is usually more expensive than sanitary prophylaxis in the long run and also prevents achieving the eradication of the pathogen from its animal reservoir, as some vaccinated individuals can remain healthy carriers. It is thus reduced to a minimum in many industrialized countries (e.g., to control FMD), although vaccination had the advantage of reducing the use of antibiotics, and thus the risk of an increasing antibioresistance.

However, vaccinations are still practiced in some developing countries, where they effectively reduce the burden of diseases, such as anthrax, contagious bovine pleuropneumonia, or FMD.

In the past two decades, the surveillance of animal diseases has been facilitated and boosted by the development of many biological tests and benefited from the molecular biology revolution, which has successively made tests, such as ELISA serology, the use of monoclonal antibodies, and finally, gene amplification, using the polymerase chain reaction (PCR) available to health authorities.

Through the introduction of these techniques, diagnostics have become more rapidly available at a lower cost and with a higher accuracy and precision. They also allow, in many cases, to assure the traceability of contamination, thus avoiding new outbreaks. During the last decade, such progress, in association with standard virus isolation, allowed rapid identification of very severe viral zoonoses, such as Hendra virus in horses in Australia, Nipah virus in humans and pigs in Malaysia, or the severe acute respiratory syndrome (SARS) in the People's Republic of China.

Similarly, the control of these diseases has benefited from very important technological progress that has been made in recent years. Vaccines with serological markers where a distinction can be made between infected and vaccinated animals have been developed, mainly for viral infections, but they are also starting to emerge for bacterial infections. The use of such vaccines allows the combination of sanitary and medical methods of prophylaxis for some diseases, such as FMD or brucellosis.

The control of certain animal diseases is at times difficult in wildlife populations, as their hosts are inaccessible to human interventions. The strategies of sanitary prophylaxis, based on the limitation of these populations, encounter technical, and, even more so, ethical problems. Oral vaccination strategies, which were able to eradicate wildlife rabies in many European countries and in North America, were developed in the 1980s using both traditional vaccines and genetically modified rabies strains [16]. These techniques are now in the development stage for two bacterial zoonoses: tuberculosis, especially in badgers [7], and brucellosis in bison, elk or wild boars [6].

In order for the veterinary authorities of its Member Countries to be informed of the most recent development in the field of these new technologies, the World Organisation for Animal Health prints and displays on its Internet web site (www.oie.int) a *Manual for Diagnostic Tests and Vaccines*, updating the diagnostic and vaccination methods recommended for animal disease control [15].

2.6 CONCLUSION

As stated in Section 2.1, it is clear that animal diseases do not present the same danger today as they did a century ago, despite some recent fears from bioterrorism threats.

The scientific progress achieved during the last century was a key factor in the extinction of this danger. The development of hygiene and asepsis and then the discovery of vaccines, later on followed by the discovery of sulfones and antibiotics, have ended the ancestral scare of animal killers, such as the rinderpest virus, or human killers, such as the glanders, tuberculosis or brucellosis agents.

In the field of food hygiene, a more rigorous control of production chains or transformation of food has considerably reduced the risk of food-borne infections. More in-depth genetic analysis of bacterial isolates also allows tracing back the origin of these infections, sometimes avoiding their diffusion from a common source.

In the middle of the last century, a strong network of international organizations dedicated to the surveillance and control of animal and human infectious diseases was established, at a regional or a global level. Such concerted actions have better chances to succeed than when organized solely at the national level, as they allow a reduction in prophylaxis costs, and specifically an improvement in the overall results by avoiding new contamination of a country by another. In addition, international cooperation programs may more easily receive financial, material, or technical aid than national programs, and they can benefit from the advice of the best international experts.

Even though the emergence that is to be most feared today is that of the resistance of bacteria to antibiotics, medical and veterinary authorities should remain extremely vigilant concerning emerging animal diseases and zoonoses, particularly in wildlife, where many pathogens may find a refuge when their domestic hosts are protected by vaccination or chemoprophylaxis.

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CHAPTER 3

HIV/AIDS Infection in the World with a Special Focus on Africa

C. Laurent, M. Peeters, and E. Delaporte

Unit of Research 145 "VIH et Maladies Associées," UMR IRD/UM1, Institute of Research for the Development (IRD), and Department of International Health, University of Montpellier BP 64501, 34394 Montpellier Cedex 5, France

3.1 INTRODUCTION

HIV/AIDS represents the prototype of an emerging disease with its worldwide dramatic consequences. The Acquired Immune Deficiency Syndrome (AIDS) is caused by two lentiviruses: the human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) [3,6]. AIDS was first recognized in the 1980s and is presently the leading cause of death in developing countries. It is believed that 40 million individuals have been infected with HIV of which about two-third live in Sub-Saharan Africa [57].

It is increasingly evident however that the virus was present in humans many decades previously when the conditions required for its epidemic dissemination were not present [9]. Recent phylogenetic analysis of different strains of HIV-1 suggests that the pandemic originates from Central Africa, two different methods pointing to the 1930s [23,48]. The oldest known HIV-1-antibody positive serum dates from 1959 and comes from the Democratic Republic of Congo [69]). HIV-2 was initially identified in two patients from West Africa, and the oldest antibody positive sera also comes from a survey done in West Africa in 1965–1969 [51].

It is likely that the human infection is due to zoonotic transmission from chimpanzees (*Pantroglodytes troglodytes*) in the case of HIV-1 and sooty mangabeys (*Cercocebus atys*) in the case of HIV-2 [15]. The passage of these viruses is readily explained by the close contact between monkeys and humans in this part of the world, and especially by hunting and butchering primates for consumption of their meat. It is very probable that sporadic isolated cases occurred on several occasions, over several decades, without provoking an epidemic. The epidemic was probably the consequence of profound social upheavals in the 1970s, combining massive urban migration, poverty, civil wars, and, as a corollary, sexual promiscuity [17,40,50].

The patterns of spread to HIV-1 and HIV-2 are highly dissimilar. In particular, the HIV-2 epidemic seems to have stabilized and may even be declining. In addition, the HIV-1 epidemic itself is geographically heterogeneous. Knowledge of the multiple factors that explain this heterogeneous spread is important for the prevention of the epidemic.

Another characteristic of HIV is its very high genetic diversity. In this chapter, we will examine how complex and evolutive the variability of HIV-1 is and analyze its consequences. Finally, we will make the point on the access of treatment in the developing world in 2005.

As Africa is the continent far most severely affected by the HIV pandemic, this chapter will be focused on this continent.

3.2 CURRENT STATE OF THE EPIDEMIC

3.2.1 Prevalences and Incidences in the World

At the end 2005, it was estimated that 40.3 (36.7–45.3) million people are living with HIV, according to the UNAIDS report [57] (Fig. 3.1). Africa represents 70% of all infections worldwide. More than 25 million Africans are now living with HIV, of whom 55% are women. The second area with highest

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

Fig. 3.1. Adults and children estimated to be living with HIV in 2005.

prevalences is Southeast Asia with more than 7 million infected persons.

In Africa, 24 million infected persons are aged between 15 and 49 years, and 1 million are children. The overall prevalence among adults in this part of the world is estimated at 8.8%, but regional variations are such that this figure has little practical value. In general, it is in southern Africa that the highest level (≥15%) is observed among the general adult population, especially in Botswana, Lesotho, Malawi, Namibia, South Africa, Swaziland, Zambia, and Zimbabwe. In Botswana, the country most directly affected, levels are particularly alarming: 40% of pregnant women living in the capital are infected with the virus, and so are 60% of patients with other sexually transmissible infections.

High prevalence rates (10–15%) have also been reported in countries such as Cote d'Ivoire, Ethiopia, Djibouti, Kenya, Central African Republic, Burundi, Rwanda, and Mozambique.

In countries such as Ethiopia, Ghana, Cote d'Ivoire, Togo, and Zimbabwe, more than two-thirds of prostitutes living in large urban centers are seropositive.

It is remarkable that although the first known HIV infections occurred in Central (HIV-1) and West (HIV-2) Africa, and in contrast with the dynamic of the epidemic in the Great Lakes area, the epidemic is recent and explosive in southern Africa where prevalences, low in 1980s except in Zambia and Zimbabwe, increased rapidly in the 1990s, making now southern Africa the leading affected part of the world.

In addition to the UNAIDS surveillance system, data from epidemiological surveys are helping to determine the epidemiological profile of HIV infection in providing complementary figures. Thus, these surveys highlight large differences in prevalences between regions, between rural and urban areas, and between population subgroups.

Recently demographic surveys using the cluster sampling method have been proposed. They provided new informations suggesting that the UNAIDS sentinelle surveillance system had overestimated the HIV prevalences.

HIV-2 has a far more restricted geographical distribution. Its epicenter is in West Africa, but more or less sporadic cases are also reported in Lusophone countries such as Angola and Mozambique [51]. The highest levels were found in Guinea Bissau, with 6.8% in a survey of semiurban areas in 1996 and 4.6% among pregnant women from Bissau, the capital, in 1997 [25]. Levels are far lower in other West African countries, ranging from 0.5% to 1.6% among pregnant women [51]. Contrary to HIV-1, the highest HIV-2 prevalence rates are in elderly people [25,51].

Incidence rates, which are used to study the dynamics of the HIV epidemic, show general upward trend, with the noteworthy exception of Uganda, where the incidence fell from 0.8 to 0.5 per 100 person per year in rural areas between 1990 and 1996 [22]. In South Africa, in contrast, the incidence in pregnant women residing in rural areas rose from 4 to 10 per 100 person per year between 1992 and 1997 [65].

Contrary to HIV-1, the incidence rate of HIV-2 infection tends to be stable, and is even falling in Guinea Bissau for example [25,51].

In Southeast Asia, the epidemic is also very heterogeneous as illustrated in Figure 3.2. The dynamic of the epidemic is also complex. In Thailand, for instance, the incidence rate is now declining, whereas in more recent infected countries such as Myanmar, the incidence rate are increasing.

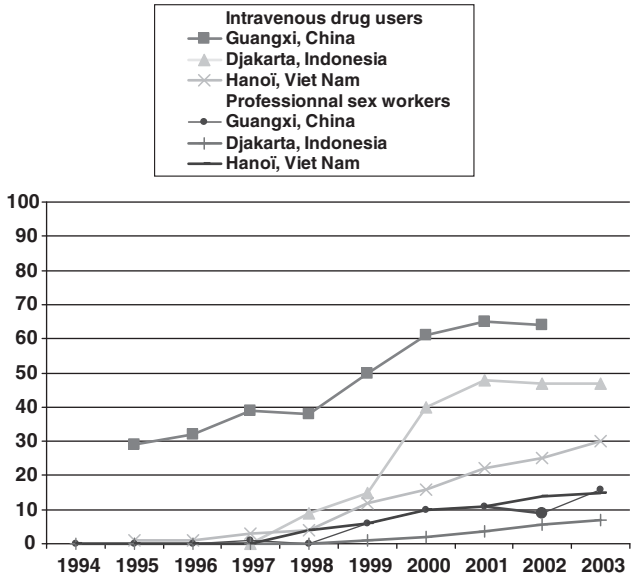

Fig. 3.2. HIV-1 prevalence rates among intravenous drug users and professional sex workers in Asia (1994–2003).

In China, the epidemic is recent but explosive. It is one of the countries where the HIV incidence is highest in the world. For instance, in Guangxi, the HIV prevalence among intravenous drug users (IVDU) is reaching 65%.

In industrialized countries, the high incidence initially observed among men having sex with men (MSM) and IVDU is now globally controlled, but the recent increase of sexually transmitted infections (STI's) among MSM is a concern. In Western Europe, the incidence rate is increasing in the heterosexual population, which is partly explained by a high proportion of migrants from Africa.

In Eastern Europe, especially in Russia or Ukraine, the epidemic is beginning to explode. For instance, the HIV prevalence rate is reaching 100% and 10% among IVDU in St Petersburg and Moscow, respectively. Predictions vary widely, but some estimate that by 2020, 14.5 million persons would be infected if denial remains the only strategy in Russia! [10].

3.2.2 Mode of Transmission

The HIV virus can be transmitted by sex, blood products, or from an infected pregnant woman to her infant.

In Sub-Saharan Africa, the predominant mode of transmission is heterosexual intercourse. Despite the same major mode of transmission, there are extraordinary differences in the spread of HIV infection among African countries, for reasons that are complex, multiple, and poorly documented.

In general, the risk of transmission depends on the infectivity of the index person, the type of sexual intercourse, and the susceptibility of the person thus exposed.

Many longitudinal epidemiological studies and direct studies of factors favoring genital HIV carriage have identified parameters influencing infectivity. One major factor is viral load in peripheral blood, as recently demonstrated in Uganda [41].

Factors leading to genital inflammation and/or infection are also important. STIs, whether or not they cause ulceration, clearly favor HIV transmission [11]. In particular, the role of herpesvirus type 2 has clearly been shown [7]. Imbalances in the vaginal flora also influence the risk of transmission [54]. A wide variety of intravaginal practices such as "dry sex" which are likely to cause irritation and disruptions of the genital mucosal epithelium have been described in Sub-Saharan Africa, but there is conflicting evidence for an association between these practices and HIV infection [49]. Concerning sexual practices, multiple partnership is clearly a risk factor, but a study comparing populations living in areas with low and high incidence rates showed no significant behavioral differences [5]. In contrast, this study, and a meta-analysis, showed that male circumcision was clearly a protective factor [63], and this was recently confirmed through a prospective interventional study comparing the incidence rate between circumcised and uncircumcised men [2]. No genetic factor specific to African populations has yet been demonstrated, either in co-receptors or the HLA system.

Among the other factors involved in the risk of transmission, the role of viral factors has been suggested [7]. Indeed, subtype C (see paragraph on HIV diversity) is associated with an explosive epidemic in eastern and southern Africa. This subtype appears to have biological particularities that could favor transmission. Several studies have shown that the phenotype of this subtype is preferentially non- syncytium inducing (NSI), implying that the asymptomatic phase, and thus the contagious period, could be longer. In addition, NSI strains use the CCR5 co-receptor present on macrophages in the genital mucosae (contrary to lymphotropic SI strains), and this could potentially favor sexual transmission of the former.

The control of mother-to-child transmission is a major challenge in Africa as in the rest of the developing world [31]. Indeed, without preventive measures, the HIV-1 transmission rate is 25–40% according to various studies versus less than 2% with adequate prophylactic measures [67]. By comparison, the mother-to-child transmission rate of HIV-2 is below 5% [51].

Two-thirds of these transmissions take place at the end of pregnancy, during delivery or very early after birth, and the viral load in the mother's peripheral blood is the main risk factor. Hence, the importance of prevention with antiretroviral drugs. Various trials have assessed the most feasible and cost-effective strategies based on the use of ZDV alone, ZDV combined with 3TC, or nevirapine monotherapy [8, 14, 29, 46, 64]. Some of these studies were based on antiretroviral treatment of the mother only, but some also included treatment of the child for a few days or weeks. Preventive efficacy is 30–50%, compared with 68% using the lengthy, complex, and costly regimens prescribed in industrialized countries. A single oral dose of nevirapine for mother and child was the strategy with the best cost-effectiveness, but the emergence of resistance is a real problem [20] and so this regimen alone is not still recommended. Recent studies in Thailand have shown the benefit of combined ARV drugs for the PMTC infection [24]. Maternal breast-feeding is a supplementary factor, doubling the risk of transmission. Nearly 75% of cases of postnatal transmission occur within 6 months after birth, as demonstrated by a clinical study in Kenya [34]. Importantly, the promotion of formula feeding in Africa must take into account the nutritional and infectious context, the poor hygiene, and the risk of stigmatization.

The importance of parenteral transmission is difficult to evaluate in developing countries. According to WHO estimates, it accounts for approximately 5% of all new cases. The main causes are blood transfusion, as transfusion safety leaves much to be desired, especially in rural areas. This mode of transmission also involves inadequate (or absent) sterilization of reusable injection materials. Some specialists consider that this has played a major role in the initial spread of the virus in Africa.

3.2.3 Impact of HIV Infection on Other Endemic Diseases

The spectrum of endemic diseases in developing countries is extensive, so we will focus on the other two main diseases, tuberculosis and malaria.

Tuberculosis is the leading cause of morbidity and mortality of HIV-infected people in both urban and rural areas.

HIV-infected patients with tuberculosis have a shorter survival and a higher tendency to acquire new opportunistic infection. For instance, active tuberculosis was present at autopsy in half HIV-1-positive cadavers in Nairobi and Kenya [42]. The risk of developing active tuberculosis among persons co-infected with HIV-1 is 5%–15% per year [43]. Thus, the AIDS epidemic is also a powerful factor facilitating the spread of tuberculosis. Several randomized controlled trials have now demonstrated that preventive therapy against tuberculosis is effective in preventing tuberculosis in HIV-infected individuals. However, feasibility studies have showed that this intervention is complex and rather inefficient [68]. Malaria is also one of the most common infections in Sub-Saharan Africa. In most ancient studies, no interaction between these two infections has been documented. However, in Malawi, it has been reported that postnatal mortality in HIV-infected infants was greatly increased in case of placental malaria infection [4]. Furthermore, in a recent cohort study performed in rural Uganda, it has been demonstrated that HIV-1 infection is associated with an increased frequency of clinical malaria and parasitaemia [66]. Taking into consideration the frequency of HIV and malaria, theses interactions could have important public health implications.

3.2.4 Demographic, Social, and Economic Consequences

AIDS is now the leading cause of death in Africa, with 3.1 million deaths in 2004 (twice the number of deaths due to malaria!) and a total of more than 17 million since the beginning of the epidemic [57].

Thus, in eastern and southern Africa, mortality rates, which had been declining in the last decades, have doubled or tripled in the last 15 years [56]. It is estimated that in 2025, the population in the 20 hardest hit African countries will be 30–120 million lower than it would have been in the absence of the AIDS epidemic [53].

Contrasting with the higher HIV prevalence rate in women, AIDS-related mortality rate is higher in men.

Infantile mortality is also affected in highly endemic countries, where all the gains made before the 1980s have been lost [53]. In southeast African countries, AIDS is responsible for up to 74% increase in deaths among children under 5 years of age, with a mortality rate of up to 30 per 1000. In a study in Uganda, the median survival time of an infected child was 21 months. As a result of the epidemic, the population of several African countries (Botswana, South Africa, and Zimbabwe) has started to decline [45]. The impact on life expectancy has also been assessed. It is estimated that each 1% rise in the prevalence rate in the general population cuts the overall life expectancy by a year.

In the next two decades, the standard age pyramid will be deeply modified by the AIDS epidemic in the countries most severely affected, with an abrupt broadening at around 20 years and a rapid decrease in the 20–40 category.

The social consequences of the epidemic are evident. In particular, the cumulative number of orphans due to AIDS in Sub-Saharan Africa increased to 12.1 million, representing 90% of all orphans in this part of the world. In Zimbabwe, 7% of all children are orphans because of AIDS.

The epidemic also has major consequences for education and general development. In Zambia, for example, more than 1300 teachers died in 1998, representing two-thirds of all teachers trained annually. The epidemic affects every socio-professional strata. As most adults fall ill during their most productive years, the economic consequences for households, enterprises, and states are considerable. Excess health expenditures are also a major burden in these countries: 40% of beds at Kenyatta hospital in Nairobi are occupied by people with AIDS, and this figure reaches 70% at Prince Regent hospital in Bujumbura [1].

3.3. MOLECULAR EPIDEMIOLOGY

3.3.1 Classification of HIV

3.3.1.1 HIV-1 One of the major characteristics of HIV is its extremely high genetic variability, which is the result of the high error rate, the recombinogenic properties of the reverse transcriptase enzyme [19], and the fast turnover of virions in HIV-infected individuals [8].

The greatest genetic diversity of HIV-1 has been found in Africa. Phylogenetic analysis of numerous strains of HIV-1, isolated from diverse geographic origins, has revealed the distinct clades of viruses, which have been named groups M (Main), N (New or non-M, non-O), and O (Outlier). Each of the three HIV-1 groups is thought to represent independent cross-species transmissions with a closely related virus. Thus, based on inferences from phylogenetic tree topologies, HIV-1 originated from simian immunodeficiency virus (SIVcpz) from *Pan troglodytes troglodytes* chimpanzees in West-Central Africa [15,47].

The vast majority of strains found worldwide belong to the group M. Within group M, there is further phylogenetic structure, which has allowed the classification of strains into subtypes.

The subtypes are approximately equidistantly related with difference of 25–35% amino acid sequence in their ENV proteins. To be considered as a subtype, isolates should resemble each other across the entire genome. In this light, there are only nine subtypes of HIV-1 group M (A, B, C, D, F, G, H, J, K) because the viruses of subtypes E and I have been found to be recombinants. Within some subtypes, further distinct sequence clusters exist, leading to the classification into sub-subtypes. For instance, subtypes A and F are subdivided into two, A1 and A2, and F1 and F2. It is clear that subtypes B and D would be better considered as sub-subtypes of a single subtype, but for historical reasons, it is difficult to change these designations (Fig. 3.3).

As our knowledge of HIV sequences improved over time, it became clear that some isolates clustered with different subtypes in different regions of their genome in phylogenetic

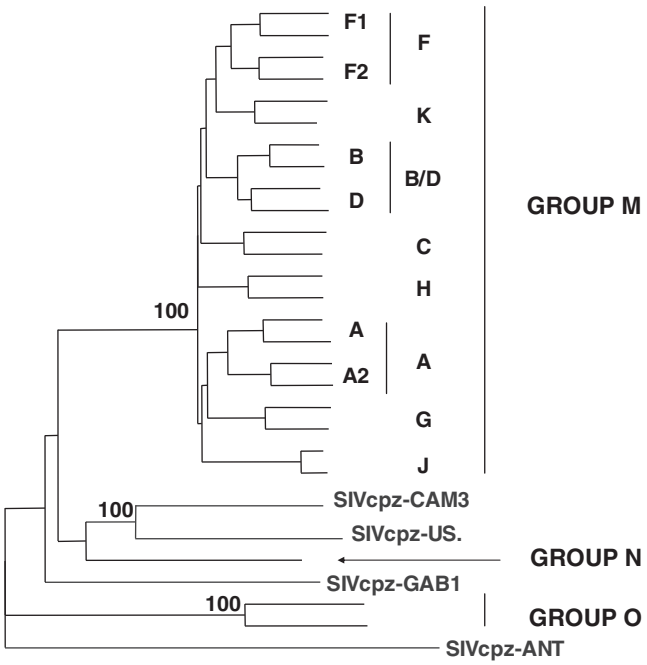

Fig. 3.3. Neighbor-joining phylogenetic tree of near full-length genome sequences of representative HIV-1 isolates from group M subtypes and sub-subtypes, group O and group N. Branch lengths are drawn to scale (the bar indicates 10% divergence). The numbers at the nodes indicate the percent bootstrap values supporting the cluster to the right (only values >80% are shown).

tree analyses [44]. Presently, some of these mosaic HIV-1 genomes play a major role in the global AIDS epidemic and are now designated as "Circulating Recombinant Forms," or CRFs. By definition, CRFs should resemble each other over the entire genome, with similar breakpoints reflecting common ancestry from the same recombination event(s): 16 CRFs of HIV-1 exits, each is designated by an identifying number, with letters indicating the subtypes involved, the letters are replaced by "cpx," denoting "complex" if more than two subtypes are involved (Fig. 3.4).

3.3.1.2 HIV-2 strains Compared to HIV-1, only a limited number of HIV-2 strains have been genetically characterized. Close phylogenetic relationship and similarities in the organization of the viral genome indicate that HIV-2 is also a result of a zoonotic transmission from SIVsm from sooty mangabeys to humans in West Africa [15]. The natural habitat of sooty mangabeys coincides with the geographical region where HIV-2 is prevalent in West Africa, and sooty mangabeys are regularly hunted for food or kept as pets, thus allowing direct contact between mangabeys and humans. More detailed phylogenetic analysis showed even that cross-species transmissions from SIVsm to humans occurred on several occasions [15]. Several of the HIV-2 subtypes have only been found in countries where sooty mangabeys are present in large numbers. Seven subtypes (A–G) of HIV-2 have been described so far. Only subtypes A and B are largely represented in the HIV-2 epidemic, with subtype A in the western part of West Africa (Senegal, Guinea-Bissau) and subtype B being predominant in Ivory Coast [22, 25, 30, 65]. The other subtypes have been documented in one or few individuals only. The different clades of HIV-2 must be the result of multiple independent cross-species transmissions of SIVsm into the human population [17]. Based on genetic distances, HIV-2 subtypes correspond to what is considered as groups for HIV-1.

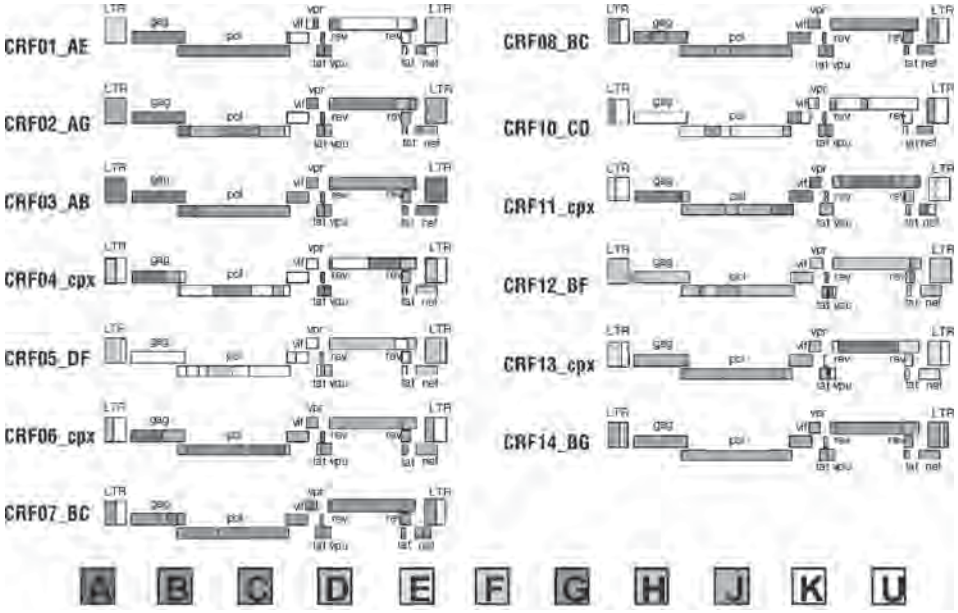

Fig. 3.4. Mosaic genome of different Circulating Recombinant Forms (CRFs). See color plates.

3.3.2 Distribution of HIV-1 in Africa

The classification of HIV strains has helped in tracking the course of the HIV pandemic [52]. Extensive efforts have been made to collect and characterize HIV isolates from around the world and Africa, and a broad picture of the distribution of HIV strains has emerged. As mentioned above, HIV-2 is restricted to West Africa, and the prevalences remain low and are even decreasing in some areas [51]. HIV-1 group O seems to be endemic in Cameroon and neighboring countries in West-Central Africa, and represents only about 1%– 5% of HIV-1 -positive samples in this region [37]. Elsewhere in the world, group O viruses have been identified mainly from persons with epidemiological links to Central Africa, mainly Cameroon and some neighboring countries. Interestingly, group N viruses have only been identified in a limited number of persons from Cameroon only [52].

The global pandemic is due to HIV1 strains belonging to group M. The distribution of the different HIV-1 group M variants in the world is summarized in Figure 3.5.

In Africa, subtypes A, C, and CRF02-AG are most frequent, but the distribution of the different HIV strains is very heterogeneous [38,39].

All groups and subtypes are found consistent with this continent being the source of the epidemic. As expected, given the presence of numerous co-circulating subtypes, a high frequency and a wide variety of recombinants have also been reported in Africa. In South and East Africa, subtype C predominates. In West and Central Africa, as judged by *env* sequences, subtype A-like viruses are most common. Full-length genome sequences of env subtype A viruses from West Africa, Senegal, Cote d'Ivoire, and Cameroon, showed that these viruses have the same recombinant structure involving subtype A and G as CRF02-AG viruses, and it seems likely that the majority of viruses with subtype A *gag* and/or *env* sequences in West Africa and West Central Africa belong to this CRF [55]. In contrast, in East Africa, the subtype A viruses are predominantly nonrecombinant. Subtype D is present at frequencies of 5–40% in Central and East Africa, whereas subtype G has been documented in many West and Central African countries. Subtypes F, H, J, and K as well as CRF01-AE, are mainly seen in Central Africa.

In occidental countries, the epidemic is mainly due to the subtype B, but there is an increasing number of non-B-subtypes in Western Europe; for instance, in France, it is estimated that about 25 % of the new infections are due to non-B-strains. In Eastern Europe, the initial infections were due to subtype B (among intravenous drug users) and subtype A (heterosexual contamination), and it is now a CRF A/B which is predominant. In South east Asia, the CRF01-AE is predominant, whereas in China, subtypes B and C and now a CRF0- BC are circulating.

In addition to CRFs, which play a major role in the global epidemic, many unique recombinant viruses have also been documented. Because only few systematic studies have been conducted, the exact prevalence of recombinant strains is unknown. Based on preliminary data, the proportion of discordant subtypes between *gag* and *env* vary from <10% to>40% according to the countries or regions studied. Peeters et al. [39] illustrate the estimated prevalences of unique recombinant HIV-1 viruses based on discordant subtype/CRF designations in different regions of the genome. The subtypes involved in these discordant samples depend on the subtypes that co-circulate in the region. For instance, in Nigeria, only subtypes A and G co-circulate, and these are the only subtypes involved in the 37% discordant samples. As expected, because subtypes circulate concurrently, a wide

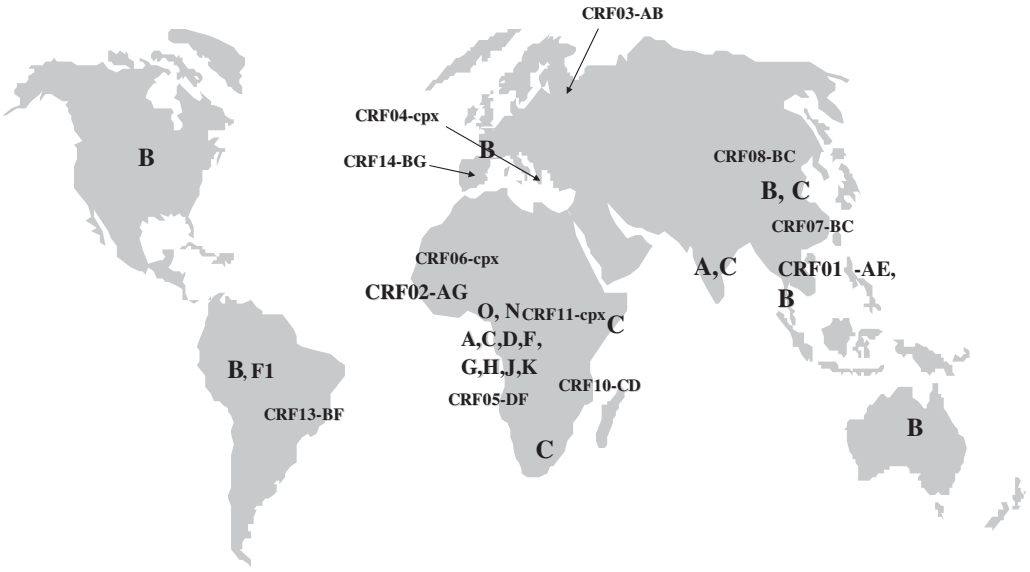

Fig. 3.5. Geographical distribution of HIV-1 subtypes and CRFs.

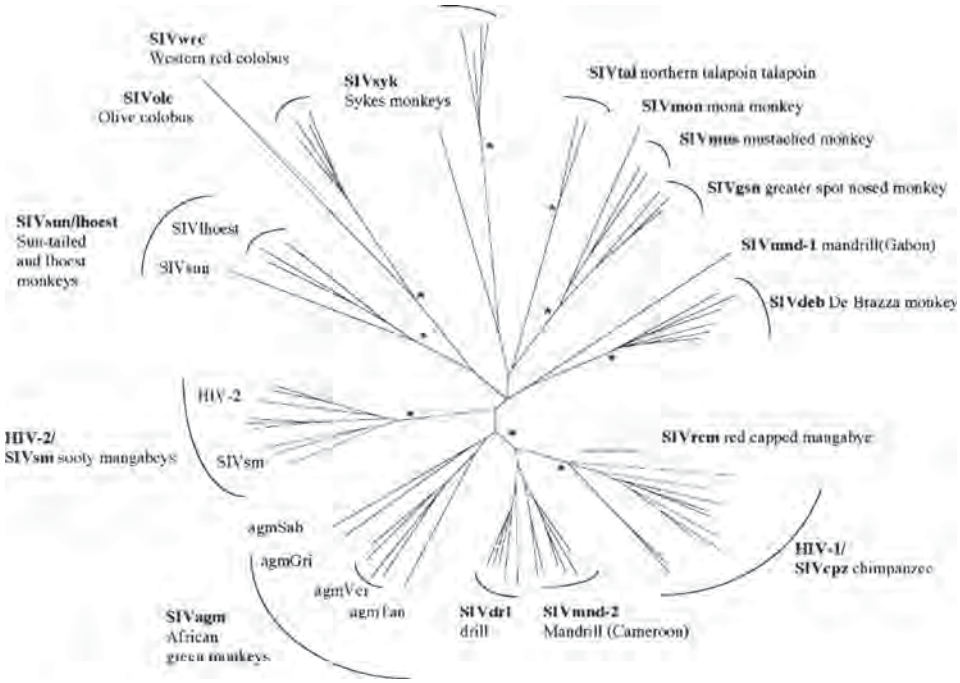

Fig. 3.6. Evolutionary relationship of a large number of SIVs characterized in *pol* region.

variety of recombinants have been reported in the Democratic Republic of Congo with all subtypes involved in the recombination events [61].

3.3.3 Implications of Recombination

As more HIV-1 variants inevitably intermix in different parts of the world, the likelihood of generating new recombinant viruses will increase. Therefore, the global distribution of different forms of HIV-1 will continue to be a dynamic process. Mosaic genomes will become even more complex, as recombination involving viruses that are already recombinant will occur. Mosaics involving CFR02-AG have already been observed in various African countries. Recombination between two CRFs (CRF02/06) has also been described in a study in Niger [28]. Even distantly related viruses have been shown to recombine. For instance, intergroup recombinants between group O and M HIV-1 strains have been documented in Cameroon (Fig. 3.6) [36]. Recombination between strains from distant lineages may contribute substantially to new HIV-1 strains and could have important consequences. Presently, group O viruses represent a minority of the strains responsible for the HIV-1 pandemic. However, if these recombinant intergroup viruses have a better fitness than the parental group O viruses, their prevalence may increase rapidly with consequences on their serological and molecular diagnosis and treatment because differences among susceptibilities to certain antiretroviral drugs have been observed *in vitro*.

Both HIV-1 and HIV-2 are of zoonotic origin, and the current HIV-1 group M pandemic provides compelling evidence for the rapidity and the extraordinary impact that can result from even a single primate lentiviral zoonotic transmission event. We recently showed that humans come in frequent contact with primates in many parts of Sub-Saharan Africa [35]; thus, raising the possibility of additional zoonotic transmission. Figure 3.6 illustrates the diversity of the primate lentiviruses actually described and to which humans are exposed through hunting and handling of bushmeat in Africa. The fact that distant HIVs can recombine begs the question whether distantly related SIVs and HIV can potentially recombine, particularly in individuals who are HIV positive and exposed to SIV by cross-species transmission. Distantly related SIVs can so spread more efficiently into the human population.

Finally, recombination also has important implications for vaccine strategies based on live-attenuated viruses, because these could recombine with infecting strains, even though the two may be quite divergent.

3.4 IMPLICATION OF HIV VARIABILITY ON PATHOGENESIS, TREATMENT, DIAGNOSIS, AND VACCINE DEVELOPMENT IN AFRICA

The majority of our knowledge on HIV is based on the specific HIV-1 subtype B predominant in industrialized countries, whereas the worldwide epidemic is due to non-B-subtype. Importantly, diagnostic tests, antiretroviral drugs, and HIV-1 vaccines have so far mainly been developed for subtype B viruses. Thus, the biological implications of HIV variability are numerous, and some of them are not fully understood.

3.4.1 Impact of HIV Variability on Diagnosis

Previous studies have shown that some persons infected with certain highly diverse strains of HIV, such as group O, fail to be diagnosed accurately by some serologic tests with for consequence tha adition of HIV-1 group O antigen in anew generation oof tests. Although enzyme linked immunosorbent assays (ELISAs) and rapid tests are sensitive and specific for diagnosing persons with chronically established infections with HIV-1 group M-non-B subtypes, the challenge still remains in diagnosing persons with recent infections primarily because antigens used for the assays were based on HIV-1 subtype B strains. Presently, in developing countries, much emphasis is being placed on implementing intervention programs resulting from new research findings such as the use of ARV drugs to reduce mother–child transmission of HIV, access to ARV in general, therapy in HIV-infected tuberculosis patients, and voluntary testing and counseling. These programs require that patients be diagnosed accurately and results delivered in a timely fashion; thus, there is considerable use of serologic assays in different countries. HIV diversity being a dynamic process, there is a need to pursue the development and evaluation of HIV diagnostics tests.

Plasma viral load measurement has increased considerably in clinical settings for monitoring patients on ARV therapy. Although, much progress has been made to improve the sensitivity of nucleic-acid-based assays to quantify viral load, some RNA viral load assays still produce erroneous results with highly divergent strains. No commercial available assays exist for HIV-2 quantification.

3.4.2 Impact of HIV Variability and Antiretroviral Therapy

The HIV diversity raises two questions. First, is there a natural resistance to some ARV drugs developed for the subtype B; and secondly, the acquired resistance under drug pressure is the same as for subtypes B and non-B. Concerning the first question, HIV-1 group O and HIV-2 strains are naturally resistant to non-nucleoside reverse transcriptase inhibitors. Limited *in vitro* studies have suggested that some non-B-subtypes may be less susceptible to certain classes of ARV drugs. For instance, subtype G strains are less susceptible *in vitro* to protease inhibitors. Genetic characterization of the protease gene from non-B-strains revealed a high genetic polymorphism with minor mutations [58]. Accessory (or minor) mutations may not result in a significant decrease in susceptibility, but may be associated with an increase in viral fitness (replication capacity) and/or increase in resistance level associated with major mutations, and thus, long-term failure of therapy. However, the implication of the presence of only accessory mutations to susceptibility of ARV drugs still has to be investigated *in vivo*. Results of ARV drug initiatives in Africa (Senegal, Cote d'Ivoire, and Uganda) and studies on African patients in Europe have showed that the presence of accessory mutations in the protease gene at baseline do not influence the clinical outcome of HAART [12]. Indeed, virologic responses (decrease in plasma viral loads and increase in CD4 count) comparable to responses reported among patients infected with subtype B in Western countries have been observed in Senegal and Uganda [26,62]. Other studies have also not found subtype-dependent responses to ARV therapy for subtypes A, C, and D. However, more data and a longer follow-up are needed to identify whether pre-existing accessory mutations could influence the rate of occurrence of resistant viruses during treatment and to what extent they could compensate for the reduced fitness of resistant mutants.

As ARV drugs are beginning to be widely used in Africa, studies are needed to understand the development of ARV drug resistance in patients infected with different subtypes. Development of drug resistance may be influenced by levels of viral loads among the patients, greater quasi-species distribution, and genetic diversity. Subtype G viruses have the V82I naturally occurring polymorphism at a position where major drug-resistant mutations occur. But primary or major mutations related to ART resistance have not yet been documented as natural variants in non-B-group M stains. It is also not known whether primary mutations that confer resistance in subtype B viruses also play a role for non-B-subtypes. However, of the few published studies on ART drug resistance in Africa, a strong correlation has been documented between genotypic and phenotypic resistances, and mutations observed so far correspond to similar mutations seen in subtype B infections under similar treatment regimens, but other studies suggest that mutations associated with drug resistance might differ. An important point is that the algorithm used to interpret the observed mutations is based on B subtypes and that this algorithm is sometimes not relevant for non-B subtypes [62]. Moreover, the few studies reported in Africa have shown that the rate of occurrence of drug resistance depends largely on the appropriate use of the drugs than on the HIV-1 subtypes. For instance, more than 50% resistance has been reported in Cote d'Ivoire and Gabon among patients receiving ARV drug therapy without appropriate clinical and laboratory follow-up. In contrast, in Senegal, after a 24 months follow-up period, drug resistance mutations were seen in only 16% of the patients receiving ART with careful clinical and biological monitoring [60], which is comparable to what has been described for patients infected with subtype B viruses in Western countries. However, one study has shown *in vivo* differences related to subtypes. The study from the HIV Network for Prevention Trials (HIVNET 012 study) in Uganda showed that resistance to nevirapine occurred more frequently in women infected with subtype D than in women infected with subtypes A and C [16]. Overall, in order to avoid the rapid emergence of resistant viruses on a large scale in developing countries, it is important that infrastructure necessary to monitor responses to ART be put in place in these countries and that clinicians are trained in the appropriate use of ART drugs and continuous surveillance of ART drug-resistant viruses has to be organized to guide ARV treatment strategies and policies.

3.4.3 Impact of HIV Variability on Transmissibility and Pathogenesis

Compared with HIV-1, HIV-2 infection is characterized by a much longer asymptomatic stage, lower plasma viral loads, slower decline in CD4+ T cell count, and a lower mortality rate. The existence of many other factors that influence transmissibility and pathogenicity makes it difficult to establish the impact of HIV-1 viral subtypes. Limited studies on subtypes and transmissibility have yielded discordant conclusions. A recent study in Tanzania suggested that subtypes A and C, and recombinants are more likely to be perinatally transmitted than subtype D. On the contrary, a study in Uganda suggests similar rates of perinatal transmission for subtypes A and D, but a study in Kenya has shown that women infected with subtype D were more likely to transmit virus to their infants than those infected with subtype A.

HIV-1 subtype-specific difference in disease progression appear conflicting. For instance, no difference in disease progression was found between patients infected with subtypes B and C in Israel, or among patients infected with subtypes A, B, C, and D in Sweden. In a 4-year prospective multicenter study of 335 patients from Senegal and Cameroon with unknown dates of seroconversion, multivariate analyses showed no difference in survival, clinical disease progression, or CD4 cell decline between patients infected by CRF02-AG strains and those infected with other strains. However, two studies based on incident cases have found HIV-1 subtype-specific disease progression patterns. In a study in Uganda of more than 1000 patients, subtype D was associated with faster progression to death and with a lower CD4 cell count during follow-up, compared with subtype A, after adjusting for CD4 cell counts at enrollment.

3.4.4 Impact of HIV Variability on Vaccine Development

An effective HIV vaccine is the long-term solution to control the HIV/AIDS epidemic in Africa and should protect against infection to all genetically diverse strains of HIV-1. From a vaccine standpoint, the implication of the multitude of HIV-1 subtypes circulating in Africa is unknown, as correlates of protective immunity against HIV-1 are poorly understood. Few and contradictory data exist on the link between genetic subtypes and HIV-specific immune responses [33]. Several studies have not shown a correlation between HIV-1 subtypes and neutralizing serotypes. However, one study has reported subtype-specific neutralization for subtype B and CRF01-AE in Thailand, and one study also suggested geographically clustered neutralization sensitivities within subtype C. Because CTL are important components of the antiviral responses in HIV-infected people, current efforts on HIV vaccines are targeting induction of T-cell responses, particularly against gag and pol proteins, which appear to be more conserved in HIV. Studies have demonstrated CTL cross-reactivity between different HIV-1 subtypes to varying degrees of conservation in the genes. This may suggest that matching HIV vaccine candidates to the prevalent HIV-1 strains might be less important for vaccines targeted at induction of T-cell responses to conserved proteins (for instance, gag and pol). However, intra-subtype CTL responses are usually stronger and more frequently detected than inter-subtype reactivities, and subtype-specific CTL epitopes have also been identified [13, 20,55].

Because of the distribution pattern of HIV-1 subtypes in the world, current vaccine development efforts have been subtype specific.

3.5 ACCESS TO TREATMENT

HAART has dramatically reduced HIV/AIDS-related mortality, morbidity, and hospitalization in industrialized countries, and thus HIV/AIDS can be considered much more a chronic disease rather than a lethal disease, as we can control the replication of the virus, but its eradication in humans is not yet possible. The major public health problem is now to make effective such care for people living in developing countries, that is, for the majority of the persons who need treatment! WHO estimated that in 2005 only 8% of persons living in Africa and who need ARV have access to such treatment.

At the end of 1998, the necessary and legitimate access to antiretroviral drugs was not considered as an evidence. At that time, the cost of drugs and reagents, the need for relatively sophisticated laboratory facilities for treatment monitoring, and the infrastructure required to provide an uninterrupted supply of drugs were considered as important limitations on wide spread use of HAART in poor countries. Other hindrances include the supposed complexity of antiretroviral drug administration, drug interactions, rapid emergence of viral resistance, the frequency of adverse effects, poor adherence, and inadequate knowledge of biological and clinical responses in patient infected by non-subtype-B HIV-1 strains. Furthermore, most patients have advanced HIV disease by the time treatment is initiated, and this could lead to higher toxicity, lower efficacy, and severe immune restoration syndromes. These factors were sometimes used as a pretext for focusing public health intervention exclusively on prevention rather than prevention and treatment. The first governmental African national initiative was set up in Senegal by Dr Ibrahima Ndoye who can be considered as a visionnaire. The result of this program, followed now by numerous others, has demonstrated that the efficiency of such treatment is the same as in occidental cohort. At the same time, the drastic price reduction (90%) of brand-name drugs, the availability of generic drugs and their proven efficacy [69], and the simplification of treatment (a once a day treatment is possible) have changed the landscape for ARV in resource-poor settings. Furthermore, the United Nations global fund the World Bank and numerous other governmental initiatives have generated funds for ARV treatment as never. Despite this, the treatment remains beyond the reach of all. The reasons are multiple. It is clear that the international agencies have generated an extraordinary "bureaucracy" with

nonoperational procedure for the management of such program. In some countries, different international supports are not under the responsibility of the different structures generating conflicts. Furthermore, at the request of WHO and UNAIDS, multisectorial program including different ministries have been created. The result is that in some countries there is competition between AIDS program from the ministry of Health and the multisectorial program. This reform has generated still more "bureaucracy." Beyond these structural problems, factors implementing ARV programs are difficult not only because it is a life-long treatment that requires a good follow-up in order to avoid virological failure and acquired drugs resistance but also because it is a global health program with, for instance, the need to promote voluntary testing and counseling. In order to reach the objective of treating 3 million persons in 2005, WHO has promoted a simplified approach using mainly clinical criteria for the follow-up of the patients. Clearly, this strategy needs to be evaluated through operational research, as we have to be sure that this strategy is not dangerous for the patients in the short time (tolerance, virological failure) and for the community in the mid-term (emergence of resistance).

3.6 CONCLUSION

In the developing world, especially in Sub-Saharan Africa, the HIV epidemic is no longer only a public health problem, it is also affecting the development. A vaccine is eagerly awaited. Several candidate vaccines are now being studied, but it will take several years to develop a safe and routinely effective vaccine covering all the circulating strains. The good news is that we have an effective treatment, but in the meantime, access to antiretroviral regimens is a major concern.

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CHAPTER 4

Molecular-Phylogenetic Strategies for Characterization of Uncultured Pathogens

Daniel N. Frank[1] and Robert A. Feldman[2]

[1]*University of Colorado, Boulder, USA*
[2]*SymBio Corporation, Menlo Park, CA, USA*

4.1 INTRODUCTION

During the late nineteenth century, theories of disease transmission were revolutionized by the development of methods for isolating microorganisms in pure culture. As the etiological agents of such noteworthy diseases as anthrax, tuberculosis, and cholera were demonstrated to be microbial, the miasmatic theory of disease gradually was supplanted by the theory that disease could be caused by infectious microorganisms [5]. In 1882, Robert Koch set forth guidelines, popularly known as "Koch's Postulates," for proving that a microorganism causes a particular disease. The cornerstone of demonstrating causation in Koch's schema is the isolation and propagation of an infectious agent in pure culture. The putative pathogen must then be demonstrated to cause disease in an animal model and subsequently be re-isolated in pure culture.

Applications of Koch's postulates have succeeded spectacularly in proving the infectious etiologies of a wide variety of acute, monomicrobial diseases. But, what of diseases in which an etiological agent cannot be cultured? Notable examples include *Treponema pallidum* and *Mycobacterium leprae*, known to cause syphilis and leprosy, respectively, but which are recalcitrant to *ex vivo* cultivation. Pure-culture isolation of a potential pathogen can be a challenging proposition if it is fastidious and/or strictly anaerobic. Such microbes may be substantially underreported, or overlooked altogether, by cultivation and plate counts relative to more easily grown species. In many natural environments of complex microbial communities, less than 0.1% of viable cells can be cultivated by conventional techniques [124]. Although, in general, the success rate of cultivating human-associated microbes is substantially better than for other environments, these techniques still fail to identify the majority of microbes in any sample, even for the well-studied gastrointestinal microbiota [61,171,175].

In addition to culturability, Koch's postulates presuppose a one-to-one relationship between pathogenic species and disease. For polymicrobial diseases (e.g., gingivitis), which may involve complex consortia of microbes, it is unlikely that Koch's postulates can be strictly fulfilled. Moreover, as infectious diseases have been brought under, at least temporary, control in developed countries, chronic diseases have surpassed acute infectious diseases in importance, as measured by morbidity and mortality.

Epidemiologists now recognize that chronic diseases most often arise from a complex interplay of genetic and environmental factors, including exposure to infectious agents. Exposure to a pathogenic microbe therefore may not be sufficient by itself to cause a particular disease, but instead may represent a risk factor of some magnitude for disease progression [5]. Depending on the degree of suspicion that an infectious agent is involved in a chronic disease, appropriate microbiological tests may or may not be conducted in determining the etiology of the disease. Part of the problem is that microbes involved in chronic diseases, in contrast to acute, fulminant diseases, likely are not particularly amenable to isolation in culture, due perhaps to their paucibacillary nature, fastidiousness, or novelty. Therefore, the onus is placed on the ingenuity of clinicians

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

and scientists to determine whether and to what extent infectious agents (e.g., *Helicobacter pylori* [105]) are associated with a particular chronic disease.

Finally, any search for a putative pathogen must take place within the background of a highly complex human–microbial ecosystem. In a sense, our bodies are not predominantly eukaryotic, being colonized by an extraordinary number of microbial cells estimated to outnumber human cells by 10:1 [151]. Most of these microbes are commensal (i.e., benign) or symbiotic (i.e., beneficial) organisms, which contribute to the maintenance of the health of the human host. Because of the many benefits provided to the host by the normal, healthy microbiota, any disruption of this community, by pathogenic organisms or other means, would be expected to negatively impact the health of the host. Conversely, as the normal human microbiota is better understood, in terms of composition and ecological relationship, it may be possible to rationally manage or restore this microbiota, to bioremediate the human host in the terms of Salyers and Shipman [150], in order to prevent or treat disease. Thus, a full characterization of the ''normal'' microbial ecology of the human host (i.e., the microbial constituents and their interactions with each other and the host) is an important prerequisite for understanding the context in which disease arises.

Nearly a century after the groundbreaking work of Koch and Pasteur, the advances of molecular biology produced a set of tools and an evolutionary framework that permit the identification and characterization of microbes in complex communities, without the requirement of prior cultivation. In this chapter, we present an overview of culture-independent microbiological techniques and their application to the problem of pathogen identification. The methods discussed below are not likely to replace traditional clinical microbiological culture; rather, they are best viewed as a complementary approach that can better inform and direct cultivation of ''unculturable'' microorganisms.

4.2 A PHYLOGENETIC FRAMEWORK FOR CULTURE-INDEPENDENT PATHOGEN DETECTION

4.2.1 Molecular-Phylogenetic Analysis of Ribosomal RNA Genes

Culture-independent techniques for analysis of microbial communities most often employ nucleic-acids-based assays for detecting and characterizing the genetic compositions of microbes. Perhaps the most widely studied genes in this respect are those encoding the small and large subunit ribosomal RNA (SSU and LSU rRNA genes, respectively). Because they are present in all cellular forms of life, rRNA gene sequences can be used to map the evolutionary, or phylogenetic, relationships of all organisms. Pioneered by Carl Woese in the late 1970s, analyses of rRNA sequences produced the first truly objective

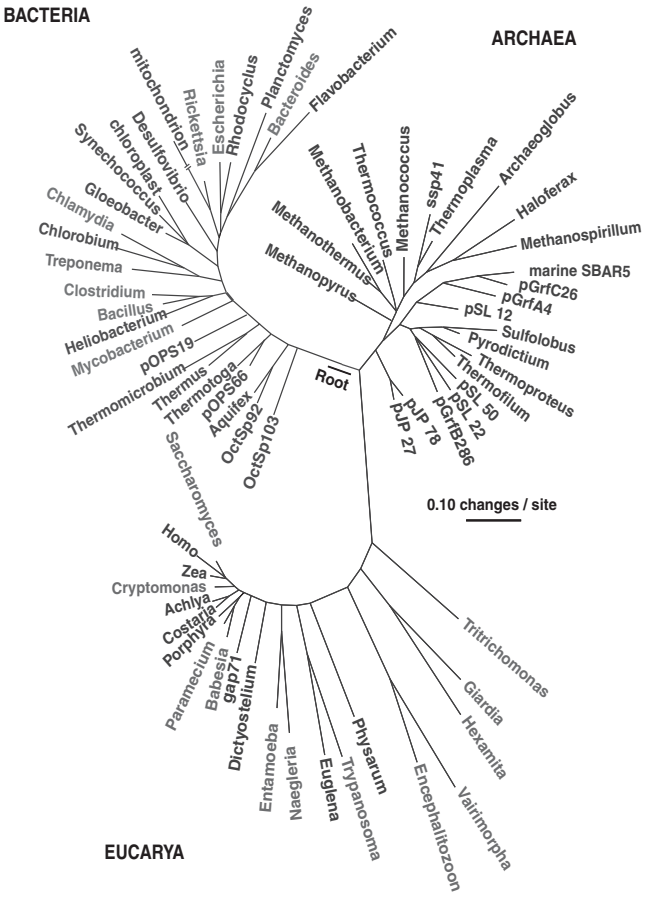

Fig. 4.1. Small-subunit RNA-based phylogenetic tree. Representatives of the three primary lines of cellular life, the Archaea, Bacteria, and Eucarya are shown. Lineages colored red represent groups harboring pathogens. Adapted from [124].

inferences of the large-scale phylogenetic organization of biological diversity [193]. Figure 4.1 (adapted from [124]), for example, shows a universal SSU rRNA-based phylogenetic tree, depicting the evolutionary interrelationships between organisms representative of the three primary lines of descent: the Bacteria, the Archaea, and the Eucaryotes. More precisely, this tree depicts the evolution of a particular gene, in this case the SSU rRNA gene. However, the extrapolation from gene-based tree to organism-based tree can be inferred with reasonable confidence because phylogenetic analyses of many information-processing genes produce congruent trees [62].

Early phylogenetic analyses utilized rRNA gene sequences that were isolated from axenically cultured organisms. However, Pace and co-workers [33,90,91,165] demonstrated that rRNA sequences could be determined from RNAs or DNAs obtained directly from the environment. Phylogenetic analyses of these environmental rRNA sequences allowed for the identification of the resident organisms, thereby bypassing the need to culture. In this manner, microbes present in complex samples (e.g., a colonized human tissue) can be incisively identified independent of culture. At first, rRNA

gene sequences were obtained by directly sequencing rRNAs extracted from environmental samples [91,122,165]. Subsequent development of the polymerase chain reaction (PCR), coupled with the identification of highly conserved regions of primary sequence within the small-subunit (SSU) rRNA, allowed for more sensitive and facile broad-range amplification of rRNA genes from complex samples [20,91,186,191]. Such culture-independent molecular methods have revolutionized microbial ecology by radically expanding our knowledge of the breadth and depth of microbiological diversity [124]. Indeed, recent surveys of a number of natural environments have revealed the existence of many previously undescribed, novel groups of Bacteria, Archaea, and Eucaryotes [4,30,34,71]. Moreover, application of these molecular techniques to clinically relevant environments have greatly extended our understanding of medical microbiology and provided several success stories of the characterization of "unculturable" pathogens (detailed below).

A general scheme for culture-independent phylogenetic analysis of mixed microbial communities is shown in Figure 4.2. In brief, mixed-community genomic DNA (i.e., DNA of both the host and any resident microbes) is extracted from a tissue sample and rDNA genes are amplified by PCR with oligonucleotide primers that recognize a broad spectrum of organisms. Libraries of rDNA genes are sorted by cloning and representatives sequenced. Comparison of the resulting sequences to rDNA gene databases and inference of phylogenetic trees indicate the identities of microbial species in the sample. A variety of oligonucleotide primers have been designed for PCR amplification of rRNA genes from specific groups of organisms [20,91,186,191]. So-called "universal" primers are predicted to hybridize to the rRNA genes of all species, thus allowing the survey of all cellular organisms in a particular sample. Alternatively, more restrictive primer sets can be used to amplify the rRNA genes of particular groups of organisms, such as genera or species.

The use of rRNA sequences presents several advantages for phylogenetic analyses of uncultured microbes. First, ribosomes are ubiquitous to cellular organisms, and so all organisms potentially are targets for molecular analysis. The use of

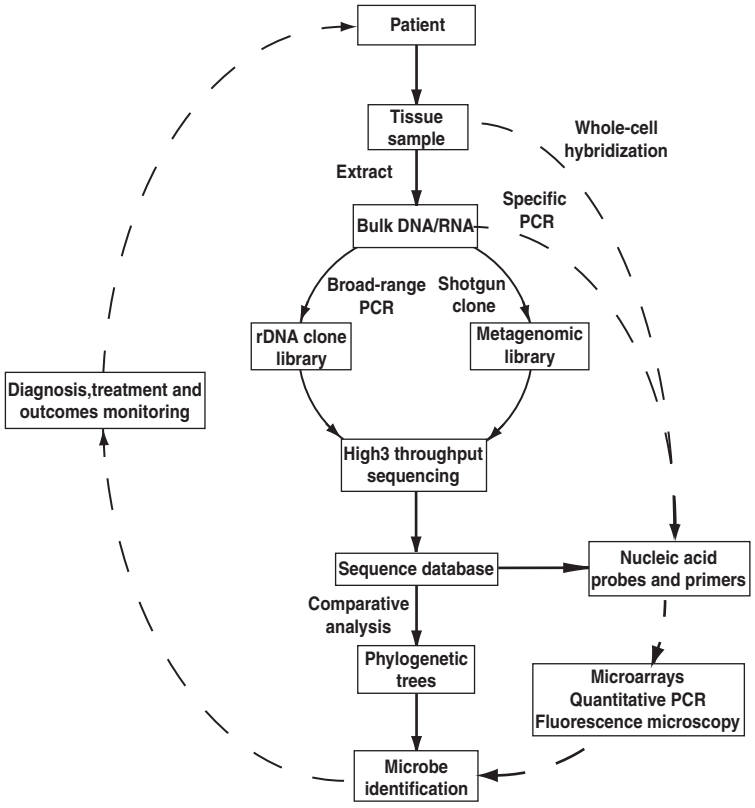

Fig. 4.2. Schematic diagram of culture-independent molecular technology applied to clinical samples. The bifurcating pathway extending from the box labeled "Bulk DNA/RNA" represents the two basic strategies for culture-independent molecular analysis described in the text. In both cases, bulk nucleic acid, which contains host and microbial DNA, is isolated from a specimen. Broad-range PCR with bacterial or universal rRNA gene primers produces libraries of individual rRNA gene clones, the sequences of which identify resident microorganisms. Isolation of bacterial genomic DNA from specimens and shotgun cloning produces metagenomic libraries, from which whole genome sequences can be assembled. The collection of sequences can be used to design nucleic-acid-based tools for microbe identification, and ultimately, more incisive treatment of the patient.

broad-range PCR primers to isolate rRNA genes from complex communities requires no *a priori* assumptions about the kinds of microbes present in a sample. Second, rRNA sequences are not prone to lateral gene transfer, which can obscure the true phylogeny, and hence accurate identification, of organisms. Third, regions of rRNA sequences evolve at different rates, making them appropriate for the resolution of taxonomic lineages ranging from species to kingdoms. Last, due to the aforementioned reasons, a database in excess of 400,000 SSU and LSU rRNA sequences has accrued over the years. Any newly acquired rRNA sequence is likely to be reasonably closely related to a databased sequence. Hence, phylogenetic analysis of a new sequence can provide incisive information about the identity of the microorganism from which the rRNA gene was isolated; in contrast to the ambiguities inherent in phenotypic or biochemical assays, the analysis of DNA sequence data provides an objective, natural system for classifying microbes.

4.2.2 Application to Monomicrobial Infections

Initial successes in applying rRNA phylogenetics to uncultured pathogens came in the study of diseases caused by single microbial species. We report a selection of these studies.

4.2.2.1 Bacillary angiomatosis In 1990, Relman et al. [137] reported the results of a broad-range 16S rRNA survey of patients with bacillary angiomatosis (BA), a potentially life-threatening systemic vasculoproliferative disease that most often affects immunocompromised individuals [77]. Despite the clear appearance of bacilli in diseased tissues (e.g., skin nodules and regional lymph nodes) and positive response of BA to antibiotic treatment, no organism could be cultured from BA lesions. However, using PCR primers conserved in all bacteria, Relman et al. [137] succeeded in amplifying 232- and 480-basepair PCR fragments from samples of spleen and splenic hilar lymph nodes obtained from a patient. Significantly, more specific primers designed on the basis of the 480-basepair sequence also produced PCR products of nearly identical sequence in samples obtained from three additional BA patients. Initial phylogenetic analysis of the cloned rRNA gene sequences indicated a close relationship of the putative BA agent with *Bartonella quintana* (formerly *Rochalimaea quintana*, the cause of trench fever), a member of the α-Proteobacteria, as well as a more distant relationship with the α-Proteobacterial genus *Rickettsia*. More detailed analysis of a nearly full-length rRNA amplicon from BA tissues confirmed this phylogenetic assessment [136]. The causative agent of BA has now been isolated and named *Bartonella henselae* [135].

4.2.2.2 Whipple's disease First described in 1907 [188], Whipple's disease is an extremely rare disorder (~12 new cases/year worldwide) characterized by diarrhea, weight loss, malabsorption, and arthropathy [37]. Historically, Whipple's disease has been confirmed by detection of non-acid fast, periodic acid-Schiff (PAS) staining inclusions in macrophages found within affected tissues [138]. Whipple reported the presence of silver-staining, rod-shaped organisms in the vacuoles of PAS-positive macrophages, a finding that has been confirmed by conventional and electron microscopy [37,188]. Successful treatment of the disease with antibiotics also lent support to the idea that Whipple's disease was caused by an infectious bacterium, despite repeated failures to isolate such an agent in pure culture.

The first clues to the identity of the organism detected by Whipple were reported by Wilson et al. [190] and Relman et al. [138], both of whom used broad-range PCR to amplify rRNA genes directly from diseased specimens, including those from the small bowel and a variety of lymph nodes. Surprisingly, given the presence of commensal microbiota in the small intestine, broad-range PCR identified only a few highly similar sequence types in the Whipple's samples, suggesting the detection of a single microbial species. Phylogenetic analysis of these sequences placed the putative Whipple's disease bacillus within the bacterial division Actinobacteria (i.e., the high G+C Gram-positive clade), but indicated that the sequences were only ~92% identical to those of known organisms. Now named *Tropheryma whippleii*, the Whipple's bacillus represents a new genus and species. Recently, *T. whippleii* has been grown from specimens of mitral valve endocarditis [132] and cerebrospinal fluid (CSF) [101] by stable co-culture with human fibroblasts. Significantly, PCR and fluorescence *in situ* hybridization (FISH) of culture samples with species-specific rRNA oligonucleotides proved crucial in tracking the propagation of the bacterium in culture. As detailed below, genomic analysis has provided several important insights into the physiology of *T. whippleii* that resulted in the recovery of this pathogen in pure culture.

4.2.2.3 Human ehrlichiosis Members of the genus *Ehrlichia* are emerging as a source of tick-borne zoonotic infections in humans. *Ehrlichia* species infect the phagocytic cells of mammals, including humans, and cause undifferentiated fever, headache, and myalgia several weeks following transmission by tick bite [36,125]. Prior to 1987, only one *Ehrlichia* species, *Ehrlichia sennetsu*, was recognized to cause human ehrlichiosis. Two studies published in 1987 reported cases similar to Rocky Mountain spotted fever, but with serological and morphological (i.e., detection of a morula, a vacuolar cluster of coccobacilli) findings suggestive of *Ehrlichial* infections [45,100].

Because *Ehrlichia* are difficult to culture and phenotypically characterize *in vitro*, Anderson et al. [1] employed broad-range PCR to identify the microbes associated with the newly recognized human ehrlichiosis. Bacterial rRNA genes were amplified from the blood specimens of two patients diagnosed with ehrlichiosis, along with a patient isolate [29] grown in co-culture with canine macrophages. Bacterial rRNA genes cloned and sequenced from each of the three specimens were reported to be identical [1].

Phylogenetic analysis demonstrated that the putative causative agent was a member of the genus *Ehrlichia*, most closely related (~98% rRNA sequence identity) to *Ehrlichia canis*. Although there is no formal rule for differentiating species based on rRNA sequence comparison, the ~2% sequence divergence between *E. canis* and the presumptive etiologic agent was greater than intraspecies rRNA sequence distances measured for other *Ehrlichia* species. Lack of serological cross-reactivity between *E. canis* and the newly identified microbe, coupled with the phylogenetic analysis, suggested that the agent of human ehrlichiosis was a novel species, named *Ehrlichia chaffeensis* [1]. Although Koch's postulates have not been fulfilled for this organism, *E. chaffeensis* is generally acknowledged to be one cause of human monocytic ehrlichiosis [36,125].

Sequences obtained in broad-range rRNA phylogenetic studies, of mixed communities or axenic isolates, constitute a valuable resource for subsequent work. Once a sufficiently broad set of sequences are determined for a particular group of organisms, group- or species-specific PCR primers can be designed and used to analyze the microbial group in greater detail. Buller et al. [18] used this approach in a clinical setting to establish the prevalence of *Ehrlichia* species in 413 patients presenting with possible ehrlichiosis. Broad-range rRNA primers with specificity for all known *Ehrlichia* species produced positive PCR results in 60 (~15%) of the samples. The rRNA sequences of 56 of the PCR positive specimens were indicative of infection with *E. chaffeensis*. However, the sequences of the other four PCR-positive specimens were identical to the rRNA sequence of *Ehrlichia ewingii* a close relative of *E. chaffeensis* previously identified only in cases of canine granulocytic ehrlichiosis. Specific PCR assays for *E. ewingii* (positive results) and *E. chaffeensis* (negative results), along with serological and morphological (i.e., detection of morulae in granulocytes, rather than monocytes) evidence, clearly indicated that *E. ewingii* was the causative agent of granulocytic ehrlichiosis in all four cases [18].

4.2.2.4 Non-Helicobacter pylori infections Spiral- or curve-shaped bacteria have long been observed in the gastric mucosa of humans and other animals [164]. However, the first isolated example of these organisms, *H. pylori*, was not reported until 1984 [106]. *H. pylori* is now recognized as the primary culprit in gastritis and peptic ulcer disease and is implicated in gastric adenocarcinoma. To date, several dozen members of the genus *Helicobacter* are provisionally identified in a number of hosts and disease states [48]. Although many of these bacteria are isolated in culture, the helicobacters are fastidious, microaerophilic organisms, and may be overlooked in the course of routine clinical microbiological workups. As such, molecular-phylogenetic analysis has proven to be indispensable in the detection, identification, and characterization of members of the genus *Helicobacter* [48,162,164].

In a systematic screening for *H. pylori* infection in patients undergoing endoscopy for upper-gastrointestinal disorders, Dent et al. [31] identified large, spiral organisms morphologically and phenotypically distinct from *H. pylori* in biopsies of the gastric antrum in three individuals with chronic gastritis. Follow-up studies revealed the presence of these spiral microbes in the gastric mucosa of additional patients with chronic gastritis [110], thus supporting the hypothesis that these organisms are a cause of a mild form of chronic gastritis. Because the observed organisms were recalcitrant to cultivation, Solnick et al. [163] used broad-range PCR to amplify bacterial 16S rRNA genes from mouse gastric samples, following inoculation with two infected human gastric samples. Two distinct 16S rRNA sequences were identified in this study, each most similar to the rRNA sequence of *Helicobacter felis*. The two rRNA sequences were only 96.6% identical, suggesting the presence of multiple helicobacter species in non-*H. pylori* gastritis samples [163]. Based on additional examination of human and animal specimens, by phylogenetic analysis of 16S rRNA and urease genes, the two types of microorganisms have been provisionally named *Helicobacter suis* and *Helicobacter heilmannii* [120]. *H. heilmannii* remains uncultured.

Detection of a bacterial rRNA sequence in a clinical sample is suggestive, but not conclusive, evidence that the particular species represented by the sequence actually is present in the sample. In the absence of cultivation, morphological criterion can be misleading, as was the case with the similarly appearing *H. suis* and *H. heilmannii*. To confirm that the rRNA sequences identified by Solnick et al. [163] truly were isolated from the spiral-shaped bacteria observed in human samples, Trebesius et al. [179] used FISH to localize microbes in human gastric biopsy samples colonized with microbes with *H. heilmannii*-like morphology. *H. suis*- and *H. heilmannii*-specific hybridization probes detected spiral-form bacteria in 71/89 (80%) and nine of 89 (10%) of samples, respectively. An additional eight of 89 (9%) of the samples were hybridized with a probe specific for a novel *H. heilmannii*-like sequence characterized in this study. Intriguingly, polymicrobial infections were detected in 6% of the samples, indicating that multiple helicobacter species may contribute to pathogenicity.

In addition to their widespread association with gastroduodenal disease, the helicobacters are implicated in a range of systemic illnesses, including arthritis, bacteremia, cellulitis, cholecystitis, diarrhea, and cancer of the gall bladder. A case in point was reported for a patient with x-linked hypogammaglobulinemia who experienced recurrent abdominal abscesses [58]. Although Gram-negative fusiform bacilli were evident in samples of pus from the abscess, no organism could be cultured. The authors used broad-range PCR to amplify bacterial rRNA genes directly from DNA extracted from pus. Sequence analysis identified one bacterial type in the sample, most closely related to *Helicobacter rappini* (97% rRNA sequence identity). No other organisms were detected by culture or 16S rRNA analysis, making it likely that this novel *Helicobacter* species was the cause of the patient's abdominal abscesses [58].

Broad-range 16S rRNA PCR also proved to be critical in the diagnosis and treatment of a child with culture-negative osteomyelitis [63]. Sequence analysis of the rRNA PCR product amplified from a lesion revealed a close relationship of the putative uncultured organism with *H. rappini* (98.8% rRNA sequence identity). Subsequent treatment with antibiotics with activity against helicobacters (ciprofloxacin and clindamycin) resolved the disease [63].

4.2.3 Application to Polymicrobial Infections

A common theme of the cases presented in the preceding section is that microbes were clearly present in diseased tissues but could not be cultured. Furthermore, the abundance of these microbes, relative to other microorganisms, was such that rRNA PCR resulted in the isolation of only one or a few sequence types, suggesting a monomicrobial infection. One of the real powers of broad-range rRNA PCR technology, though, is its ability to disentangle complex communities of microbes. Tens of thousands of rRNA clones can now be assayed in a single experiment, through high-throughput methods of cloning and sequencing [39]. A potential pathogen need not be the most prevalent member of a microbial community to be detected in a sufficiently large clone library. Moreover, microbes that might be overlooked or overgrown in traditional culture (e.g., due to slowness of growth or fastidiousness) will not be similarly underrepresented in PCR clone libraries (n.b. PCR is prone to its own biases [172,184]). rRNA phylogenetics can provide an excellent means of searching for etiologic agents of a variety of acute and chronic human idiopathic diseases hypothetically caused by polymicrobial or paucibacillary infections. Below is a survey of notable applications of broad-range rRNA PCR to idiopathic diseases encompassing a wide range of conditions.

4.2.3.1 Diseases of the oral cavity In terms of rRNA phylogenetic analysis, the oral cavity probably is the best studied niche in the microbial world [173]. Ready access, the high prevalence of oral diseases (~50% of adults have gingivitis [97]), and the appreciation that most of these diseases are polymicrobial has made the mouth a favored site of molecular-based analysis. Culture studies alone have suggested that the oral cavity hosts more than 500 microbial species [114], making this site particularly attractive for broad-range PCR. Furthermore, the hypothesized role of oral microbes in causing bacteremia, endocarditis, and atherosclerosis also highlighted the need to characterize the oral microbiota [12,60,176].

Gingivitis and periodontitis are chronic inflammatory diseases of the gums that if unchecked can lead to tooth loss. Although the entire tooth generally is covered by a complex biofilm (i.e., plaque), the subgingival plaque is believed to be the primary inducer of the host inflammatory response that is ultimately pathogenic [97]. Numerous studies have therefore examined the microbial communities of the subgingival crevice, which is a microaerophilic or anaerobic environment.

Spirochetes represent a large proportion of the microbes observed in the subgingival crevices of patients with periodontitis and are correlated with disease severity, yet most are uncultivated [97]. Prompted by these findings, Choi et al. [23] used rRNA reverse-transcription PCR with bacteria-specific primers and colony hybridization with treponema-specific probes to isolate treponemal rRNA genes from a single patient with severe destructive periodontitis. Of 6418 clones screened, 74 contained rRNA genes for spirochetes. Phylogenetic analysis placed these sequences into approximately 20 phylotypes (sequence relatedness groups), based on clustering at the 98% rRNA sequence identity level, which the authors chose as a species-level cutoff. The majority of these phylotypes were unrelated to previously characterized species of treponemas [23]. A subset of the rRNA sequences were identical to sequences identified in other patients with aggressive periodontitis [24]. In a larger survey of oral spirochetes, Dewhirst et al. [32] used spirochete-specific primers to amplify rRNA genes from 15 subjects with a range of periodontal conditions. Phylogenetic analysis of 542 clones revealed 57 phylotypes of sequences with >99% sequence identity. As was observed in previous studies, the subgingival crevice of each individual harbored multiple types of treponema. Although the most prevalent clonal type belonged to the *Treponema denticola* cluster, most of the provisional species were not represented by previously cultured organisms. Together, the unexpected diversity of treponemas associated with periodontitis raises the question of whether all or a subset of the oral spirochetes contribute to pathogenicity.

Examining a wider spectrum of microorganisms within the subgingival crevice, Kroes et al. [86] analyzed 264 bacterial rRNA clones amplified directly from a single specimen taken from a patient with a case of mild gingivitis. In parallel, the specimen was subjected to standard culture in order to compare the results of molecular and microbiological techniques. Isolates were typed by phenotypic criteria, which identified only 45% of the isolates to the species level, and rRNA sequence analysis. Direct PCR produced 59 unique phylotypes, half of which were indicative of novel species (<99% rRNA sequence identity). In contrast, the majority of the culture isolates (22/28) were closely related to known species. Direct, broad-range PCR thus revealed far greater microbial diversity in the subgingival crevice than did culture. Five bacterial divisions were represented by the combined set of phylotypes: Firmicutes (e.g., *Clostridia* sp., *Staphylococcus* sp., and *Streptococcus* sp.); Actinobacteria (i.e., high G+C Gram positives); Proteobacteria (e.g., *Haemophilus* sp., *Neisseria* sp., and *Campylobacter* sp.); Prevotella; and Fusobacteria. Surprisingly, given previous reports, no spirochetes were detected in this study, although this result may have been an artifact of small sample size, as only a single specimen was analyzed.

Additional broad-range rRNA PCR surveys of periodontitis significantly expanded both the number of individuals and the range of clinical conditions samples under study (e.g., [15,22,72,126,148,149]). Paster et al. [126], for instance,

examined subgingival specimens from 31 patients, who were either healthy or diagnosed with refractory periodontitis, acute necrotizing ulcerative gingivitis (ANUG), HIV-associated gingivitis. The 2522 rRNA sequences analyzed were clustered into 347 phylotypes (groups of >99% sequence identity), 215 (62%) of which were not closely related to known species (<99% sequence identity). Healthy subjects had only a slightly greater proportion of known species as compared to disease states (58% vs. 51% (periodontitis), 53% (refractory periodontitis), 38% (ANUG), and 55% (HIV)). This study confirmed the finding by Kroes et al. [86] that members of the *Firmicutes* (low G+C Gram positives) constituted the most prevalent known (53/132) and novel (62/215) phylotypes within subgingival plaques. However, in contrast, Paster et al. [126] identified an abundance of *Spirochaetes* in their specimens.

Overall, 29 phylotypes or species were identified in four or more diseased samples, but not in healthy controls, including the suspected oral pathogens *Bacteroides forsythus* and *Porphyromonas gingivalis*; these all represent candidate pathogens. Intriguingly, several clades (e.g., sub-clades of the *Treponema*) were overrepresented in the diseased specimens, relative to the healthy controls, but no one species or phylotype was dominant. It is likely that the entire community contributes to pathogenicity in these cases.

Most studies of periodontitis have focused on surveys of the bacteria present in the subgingival crevice. However, detection of methanogenesis within this locale has stimulated a search for methanogenic *Archaea* associated with periodontitis [9,17,143]. In a survey of 48 periodontitis patients, Kulik et al. [87] used methanogen-specific rRNA PCR primers to obtain amplicons from 37 (77%) specimens. Analysis of 46 clones from 18 of the specimens revealed only three phylotypes, which were closely related to *Methanobrevibacter oralis* and *Methanobrevibacter smithii*. Despite this low diversity, identification of the same phylotypes in multiple patients indicates that these archaeal species are widespread in periodontitis patients. No healthy specimens were assayed, however, so the roles of these microbes in pathogenicity could not be inferred. In an expanded study by Lepp et al. [95], *M. oralis*-like rRNA sequences were found to be substantially enriched in periodontitis samples. Indeed, disease severity was directly proportional to the percentage of archaeal rDNA copies in specimens. Significantly, the abundance of methanogens decreased following treatment of positive clinical outcome. Lepp et al. [95] thus make not only a highly suggestive link between methanogens and periodontitis, but provide some of the first evidence that the *Archaea* can contribute to pathogenicity [40].

In addition to periodontitis and gingivitis, numerous oral diseases have been examined by broad-range rRNA PCR, including dental caries [6], dentoalveolar abscesses [38], halitosis [78], noma lesions [127], and sialolithiasis [177]. Necrotic pulp specimens from endodontic infections were analyzed by Rolph et al. [144], Munson et al. [115], and Fouad et al. [46]. A total of 55 of 70 (79%) of specimens collected during root canal were successfully amplified with general bacterial rRNA primers. As is the case with periodontitis, the predominant bacterial divisions represented by the rRNA clones were the Firmicutes, Bacteroides, Actinobacteria, Fusobacteria, and Proteobacteria. In contrast, pathogens implicated in periodontitis, including *Treponema* sp., *Porphyromonas* sp., and *B. forsythus*, were only rarely observed in endodontic infections. Rather, genera such as *Dialister*, *Eubacterium*, *Prevotella*, and *Streptococcus* were the most prevalent in necrotic pulp [115,144]. Finally, the species richness of these endodontic samples was estimated to be 90 species, much lower than that observed in the oral cavity as a whole [115].

4.2.3.2 Genitourinary disease Diseases of the prostate, such as cancer and chronic prostatitis, cause significant morbidity and mortality. Prostate cancer is one of the most prevalent forms of cancer in males [57,116], and chronic prostatitis affects at least 50% of all males during their life spans [35]. Treatment of chronic prostatitis seldom is effective or long lasting [109]. Potential pathogens (typically uropathogens such as *E. coli* and enterococci) are isolated from only a small fraction of chronic prostatitis patients. The majority of cases (>90% of symptomatic cases) are idiopathic and classified as "non-bacterial" prostatitis (also termed chronic prostatitis/chronic pelvic pain syndrome, CP/CPPS [83]).

The inability to isolate microorganisms from most patients with chronic prostatitis does not rule out microbial etiologies in these cases. As demonstrated by Nickel and Costerton [118], microbes are detectable in biofilms within prostatic ducts of chronic bacterial prostatitis specimens. Sequestration of pathogenic species, or communities of pathogens, in biofilms would limit the number of planktonic cells present in prostatic secretions, thus hindering culture. Furthermore, the assumption that prostatitis is caused by typical uropathogens may limit the breadth of microbiological assays that are routinely employed in the diagnosis of chronic prostatitis. In fact, more detailed analyses of chronic prostatitis have revealed the presence of a number of potential pathogens, including coagulase-negative staphylococci and corynebacteria, in idiopathic prostatitis cases [35,98].

A variety of studies have used broad-range rRNA PCR to search for bacteria associated with CP/CPPS [66,79,84,85,142,174]. In an early study of CP/CPPS, Krieger et al. [85] reported the presence of bacterial DNA in 103 of 144 (77%) transperineal biopsy samples. Although skin specimens also were positive in most cases (86%), sequence analysis of prostate and control rRNA clones [142] revealed that the prostate-associated microbes were not typical members of the skin microbiota (e.g., *Staphylococcus aureus*, *Staphylococcus epidermidis*). Rather, the majority of prostate-associated sequences were from Proteobacteria, including *Aeromonas* sp., *E. coli*, *Proteus vulgaris*, and *Vibrio furnissii* [142].

In a study of both bacterial and "non-bacterial" prostatitis, Tanner et al. [174] screened expressed prostatic secretions (EPS) for microorganisms by broad-range rRNA PCR.

Unlike tissue biopsies, which sample only a small section of the prostate, EPS drains from the entire ductal system of the prostate and is thus likely to provide a more representative sampling of organisms present in a specimen. The downside of sampling EPS is that care must be taken to account for urethral contaminants; Tanner et al. [174] accomplished this by screening first-void urine samples obtained prior to EPS collection. PCR products were detected in six of eight control EPS specimens and 11/17 patient EPS specimens (six of six chronic bacterial prostatitis, three of seven CP/CPPS, two of four prostatodynia).

Analysis of rRNA sequences revealed the presence primarily of *S. epidermidis*, *Propionibacterium acnes*, *Enterococcus faecium*, and *Pseudomonas* sp. in first void urine samples. Although also identified in EPS specimens, these species were a minor component of the overall microbial communities in EPS. The predominant rRNA sequences obtained from patient EPS samples were of corynebacteria and staphylococci, in addition to *S. epidermidis*. At least 15 phylotypes of corynebacteria were identified, none of which was observed in all patient samples. Instead, each patient EPS sample harbored from one to nine species of corynebacterial and seven of the 15 corynebacterial phylotypes were identified in multiple patient samples. Similar results were reported for staphyloccal and streptococcal phylotypes. No real differences in microbiota were discernible between CP/CPPS and bacterial prostatitis, although the sample populations were small. In contrast, phylotypes representative of corynebacteria, staphylococci, and streptococci were much less abundant and diverse (0–2 species) in the control EPS samples. Overall, the study by Tanner et al. [174] indicates that complex microbial communities, composed mainly of corynebacteria and staphylococci, are associated with chronic prostatitis.

Intriguingly, difficult-to-culture corynebacteria and coagulase-negative staphylococcus were previously proposed to contribute to pathogenesis in prostatitis [98,117,141]. Although none of these studies addressed the cause and effect between presence of particular groups of microbes and disease onset, Tanner et al. [174] noted a potentially important correlation between rRNA PCR results and treatment outcomes. All three PCR positive CP/CPPS samples were obtained from patients who subsequently responded positively to treatment (antibiotics and prostate massage), whereas EPS specimens were PCR negative for the remaining four CP/CPPS patients, all of whom did not respond to therapy [174]. Broad-range rRNA PCR may, therefore, provide diagnostic utility in stratifying CP/CPPS patients based on their predicted response to antibiotic/massage therapy. Indeed, the results of this PCR assay indicate that a subset of CP/CPPS cases (e.g., those with PCR-positive EPS and suites of organisms similar to bacterial prostatitis) probably should be re-classified as chronic bacterial prostatitis cases.

4.2.3.3 Gastrointestinal disease The human gastrointestinal tract is home to an extraordinary diversity of microbes, which form a community of approximately 10^{11} microbial cells per gram of gut content. Gut-associated microbial communities are relatively stable (in the absence of mitigating factors), indicating that most microbes inhabit defined niches, rather than simply transit through the lumen [151]. Numerous functions that are beneficial to the human host have been attributable to the commensal gut microbiota, including nutrient synthesis, immune system stimulation, and inhibition of pathogen colonization [49,69,150,189]. Not surprisingly, disruption of the organisms responsible for these functions can have profound effects on the health of the host. For this reason, full characterization of the composition, dynamics, and function of the normal gastrointestinal microbiota can illuminate studies of the etiologies and pathologies of gut diseases.

The sheer complexity of the GI microbial ecosystem (estimated at >500 species [68,113]), along with the fastidious nature of many of its constituents (e.g., many anaerobes) makes the gut an ideal target for rRNA-based molecular-phylogenetic studies [49]. Wilson and Blitchington [192] and Suau et al. [171] reported two of the first molecular studies of the human GI tract microbiota, each an analysis of bacterial rRNA sequences within a single human stool sample. Wilson and Blitchington [192] identified 33 unique phylotypes among their 89 sequences, and Suau et al. [171] classified their 284 clones into 82 phylotypes. In both studies, phylogenetic analysis placed the majority of the cloned rRNA sequences within one of three phylogenetic clusters: the *Clostridium coccoides* cluster XIVa (~44% of clones [171]); *Bacteroides* spp. (~31% of clones [171]); or the *Clostridium leptum* cluster IV (~20% of clones [171]). Only approximately 25% of the cloned rRNA sequences were closely related to previously reported sequences, indicating that much gut microbial diversity had yet to be revealed. In parallel with molecular analysis, Wilson and Blitchington [192] also subjected their sample to extensive cultivation, which resulted in the isolation of only 50% of the microorganisms enumerated by microscopic counts.

Studies by Hold et al. [67] and Eckburg et al. [39] found that the major fecal microbial groups, *Clostridium coccoides* cluster XIVa, *Bacteroides*, and *Clostridium leptum* cluster IV, also were the predominant phylogenetic groups in biopsies taken from the lower GI tract. From each of three healthy adults, Eckburg et al. [39] collected a set of mucosal biopsies of nominally healthy tissue from the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Ribosomal RNA clone libraries were constructed from each biopsied tissue as well as from a stool sample. In total, 13,355 bacterial and archaeal rRNA sequences were generated in this study (11,831 bacterial, 1524 archaeal). All of the archaeal sequences were nearly identical to that of *Methanobrevibacter smithii*, suggesting a dearth of archaeal diversity in the human GI tract. In contrast, the bacterial sequences constituted 395 phylotypes (>99% rRNA sequence identity), of which 80% were most closely related to uncultured organisms. Analysis of the rRNA sequence

dataset revealed that for any individual, the mucosal biopsy samples throughout the colon were remarkably similar to one another [39]. Similar findings have also been reported by Zoetendal et al. [195] and Lepage et al. [94], who used denaturing gradient gel electrophoresis to examine colonic biopsies. Fecal samples, however, were quite different from mucosal samples, indicating that stool culture may not always be an appropriate means of characterizing gastrointestinal microbiota [39,195].

The inflammatory bowel diseases, ulcerative colitis (UC) and Crohn's disease (CD), are chronic, idiopathic inflammatory disorders of the gastrointestinal tract that are characterized by high morbidity [44,157]. Although immune and genetic factors influence susceptibility to UC and CD, the GI microbiota also clearly plays a causal role in the progression of these diseases [64,128]. Many animal models of IBD, for instance, demonstrate that disease severity is significantly reduced when susceptible animals are raised gnotobiotically [140,170]. Currently, both UC and CD are thought to arise from disruption of the normal tolerance of the mucosal immune system to gastrointestinal microbes. In this model, chronic activation of the mucosal immune system leads to pathogenesis that is mediated primarily by immune effectors.

Whether inflammatory bowel disease results from a response to commensal microbes or particular pathogens remains undetermined [65,92,183]. Because of the enigmatic nature of UC and CD, these disorders have been the subject of multiple studies by broad-range rRNA PCR [13,21,27,94,102,123,129,153,169,178,185]. Using this method, Tiveljung et al. [178] detected the presence of bacterial DNA in three of five surgical biopsies from CD patients and 0/7control specimens. Chiba et al. [21] also reported a higher prevalence of bacterial DNA in lymph follicles of CD and UC subjects, compared to controls. The results of limited rRNA gene sequencing were not suggestive of potential pathogens [21,178]. A more extensive analysis of 739 rRNA sequences amplified from 31 CD and 10 normal control biopsies (both endoscopic and surgical) similarly did not reveal a specific pathogen associated with CD [129]. Nonetheless, statistically significant differences in several phylogenetic groups, most notably facultative anaerobes, were identified in comparisons between healthy and CD biopsies from both the small-bowel and colon [129].

4.2.3.4 Bodily fluids Culture of blood and CSF is of critical importance in the diagnosis of infectious disease and so culture-negative results may lead to life-threatening delays in treatment [42,182]. However various factors, including empirical antibiotic treatment, can significantly reduce the detection frequency of viable organisms in blood or CSF [19,42,182,194]. A number of studies have therefore explored the suitability of applying broad-range rRNA techniques to clinical samples of blood [28,43,54,75,76,88,89,96,107,158, 160,161,181] and/or CSF [73,82,99,104,131,152]. In addition to blood- and CSF-borne bacteria, several studies have examined blood-borne fungi [43,81,181].

Molecular analyses of CSF have focused primarily on rapid and correct identification of meningitis pathogens. In a prospective study, Schuurman et al. [152] analyzed 227 CSF samples from patients with possible bacterial meningitis. In comparison to culture, their broad-range bacterial rRNA PCR assay performed with high sensitivity (86%) and specificity (97%). Direct sequencing of the PCR products identified the culture isolates in 22/24 samples, predominantly instances of *Streptococcus pneumoniae* and *Neisseria meningitidis*. Moreover, 6 of 30 PCR-positive samples were culture negative; sequence analysis indicated the presence of *S. pneumoniae* (three cases), *N. meningitidis*, *Streptococcus agalactiae*, and *Pantoea bivia* in these cases. Kotilainen et al. [82] reported a similar sensitivity (83%) and specificity (100%) for broad-range PCR in their analysis of 56 CSF samples. Interestingly, in both studies, *Listeria monocytogenes* was isolated from CSF but not detected by PCR. The use of more than one broad-range primer set might detect additional organisms, such as *L. monocytogenes*, and thus improve the overall performance of the PCR assay in diagnosing meningitis.

A number of bacteremic and/or septicemic conditions have been examined by broad-range PCR. Kane et al. [76] compared PCR to blood culture in 30 post-surgical, intensive care unit (ICU) patients (13 transplant, 3 vascular surgery, 5 general surgery, 9 trauma or burn) with suspected infections and 30 healthy controls. PCR results were positive in 0 of 30 controls and 15 of 30 ICU patients, including all 4 patients with positive blood cultures. Whether the 11 PCR-positive/culture-negative samples were true- or false-positives was not established by Kane et al. [76], primarily because a gold standard does not exist for confirmation of sepsis. In a study of sepsis among ICU patients, Sleigh et al. [160] assayed bacteria in 197 blood samples by PCR and culture. These cases were categorized as being true positives or indeterminate on the basis of additional clinical and microbiological evidence, such as multiple positive cultures, radiological findings, or parallel culture from CSF. With respect to the true-positive samples, PCR demonstrated a sensitivity of 83% and specificity of 83% and blood culture had a sensitivity and specificity of 75% and 85%, respectively [160]. Overall, 25 of the 46 samples that were categorized as true positives were PCR positive or culture negative, indicating that PCR provided significant diagnostic information that was not available solely through culture. Surprisingly, most of the PCR-positive/culture-negative sequences were from *Staphylococcus* and *Streptococcus* species, which would not be expected to be particularly fastidious or otherwise difficult to culture.

Globally, approximately 1 million infants under 4 weeks of age die each year of sepsis [93]. Although the incidence of acute neonatal infections is low in the developed world, morbidity and mortality are still significant. Molecular analyses of neonatal septicemia have been reported by Laforgia et al. [89], Jordan and Durso [75], and Shang et al.

[158]. In a comprehensive examination of 548 blood samples from infants admitted to neonatal ICUs with suspected sepsis, Jordan and Durso [75] compared BACTEC 9240 culture to broad-range PCR of blood samples cultured in tryptic soy broth for up to 5 h. PCR and BACTEC 9240 assays produced highly concordant results, characterized by a sensitivity of 96.0% and a specificity of 99.4%. Significantly, given that only 5% of the blood samples screened was positive for either PCR or culture, the PCR assay had a negative predictive value of 99.8%. Jordan and Durso [75] concluded that their broad-range rRNA PCR assay was an accurate means of ruling out sepsis in the majority of the presumptive neonatal sepsis cases analyzed.

The aforementioned studies indicate that in acute-care settings, the rapid and incisive data produced by broad-range or specific PCR can significantly aid diagnosis and treatment of infectious diseases. For example, the broad-range rRNA PCR assay of Jordan and Durso [75] was estimated to require 9 h, compared to 48–72 h needed for BACTEC culture.

4.2.3.5 Infective endocarditis Infective endocarditis (IE) is a life-threatening microbial infection of the valves or lining of the heart. Diagnosis can be made based on the observation of vegetations by echocardiography and positive blood culture. However, blood cultures are negative in 2.5–31% of suspected IE cases (reviewed in [3,16,47]). Negative blood cultures likely are due to the fastidious nature of many of the pathogens known to cause IE [16], making this disease an obvious target for broad-range PCR analysis.

A number of studies have compared the efficacies of broad-range rRNA PCR, blood-culture, and vegetative valve-culture in IE diagnosis [10,52,53,55,56,70,74,80, 111,119,130,146,166]. Because of the desire for a rapid diagnostic assay, most of these reports entailed either direct sequencing of PCR products, rather than clonal analysis, or simply +/− scoring. Thus, microbial diversity probably was significantly underestimated in these studies. Gauduchon et al. [52], for instance, examined 52 excised cardiac valves, 29 of which were confirmed to be IE (i.e., presence of vegetations, intracardiac abscess, histopathology). Of the 29 cases, assays for microbes were positive in 10 (valve culture), 25 (blood culture), and 27 (PCR of valve tissue) instances. Combined, blood-culture and PCR were positive in 28/29 specimens. None of the 23 IE-free controls were positive for blood culture or PCR. Ribosomal RNA sequences and blood culture isolates were reported to be identical in 21/29 IE samples. In three of 29 specimens, different species were identified by blood culture and PCR; sequencing revealed the presence of known IE pathogens, *B. henselae*, *Streptococcus mutans*, and *Streptococcus bovis*, in valve tissue. An additional three of 29 specimens with culture-negative/PCR-positive results produced evidence of *S. bovis*, *Staphylocouss cohnii*, or *Coxiella burnetii* infections. Thus, rRNA PCR demonstrated not only high specificity and sensitivity in diagnosis of IE, but altered identification of the putative etiological agent in six of 29 cases. In a similar study of IE, Greub et al. [55] reported a specificity of 100% and sensitivity of 61% for PCR of valve samples, compared to histological examination of tissues. Because multiple studies have affirmed the validity of broad-range rRNA PCR in diagnosing IE, Millar et al. [111] have suggested that the Duke's criteria for diagnosis of IE be amended to include PCR-based results.

4.3 WHOLE GENOME CHARACTERIZATION OF UNCULTURED PATHOGENS

Ribosomal RNA-based phylogenetic analyses have established a comprehensive framework for identifying and characterizing uncultured organisms. However, with respect to pathogenicity, culture-independent rRNA studies have certain limitations. Microbial genomes often are characterized by a remarkable degree of intraspecies variation in gene content, despite having nearly identical rRNA sequences (*cf.* [133,154,187]). Thus, rRNA sequences generally do not have the power to resolve pathogenic and nonpathogenic strains of the same species. In practical terms, this means that rRNA analysis is not particularly informative in conditions in which pathogenic and commensal strains occupy the same niches (for instance, commensal vs. pathogenic *E. coli* located in the lower GI tract). In these cases, an rRNA survey would simply note the presence of a microbial species without providing an indication of its pathogenic potential.

The presence or absence of the more evolutionarily variable genes within a genome, rather than variations in rRNA sequence, allow closely related species or strains to occupy unique niches. Genomic differences between pathogenic and nonpathogenic relatives often entail variability in virulence-determining loci, differences that can be detected and exploited in culture-independent molecular analyses. Clinical microbiology has fully integrated the use of PCR assays for detection of particular pathogenic species via amplification of species-specific genetic loci. Recently, however, the rapid expansion of genomic sequencing capabilities has allowed studies of microbial genomes to be carried out in a culture-independent manner, analogous to rRNA analyses. In these cases, a major challenge is to obtain the genomic DNA of the desired microorganism or microbial community in the absence of contaminating host (e.g., human) genomic DNA, which often is in great excess. Two basic strategies, both outlined in Figure 4.2, have been employed to analyze "unculturable" genomes depending on the complexity of samples.

4.3.1 Enrichment of Monocultures

Several obligate intracellular pathogens that have not been successfully grown axenically can nonetheless be propagated under conditions that permit genomic studies. Typically, these pathogens are co-cultured with host tissue culture cells, which provide suitable conditions for intracellular growth. Following expansion of these cultures, host cells are gently lysed and released bacterial cells are purified by differential

centrifugation. The pathogens *Chlamydophila caviae* (formerly *Chlamydia psittaci* [134]), *Chlamydia pneumoniae* [133,159], *Chlamydia trachomatis* [133,167], *Rickettsia felis* [121], *Rickettsia prowazekii* [2], *Rickettsia typhi* [108], and *T. whippleii* [11] all have been isolated in this manner. An alternative approach is to infect animal models with particular pathogens and then purify bacteria from infected tissues by methods similar to those used with tissue culture cells. *M. leprae* [25,41], and *T. pallidum* [51], for example, have been isolated from armadillo liver and rabbit testes, respectively.

Following isolation of pathogen cells from contaminating host material, bacterial genomic DNA is isolated, fragmented, amplified in cloning vectors, and sequenced. The whole genome sequence eventually is constructed by pasting together the sequences of many overlapping cloned genomic fragments. The application of bioinformatics and functional-genomics methodologies to the genomic sequence, which is beyond the scope of this chapter, then can begin to disentangle the genetic factors that determine the ecology of the pathogen. In many cases, the difficulties associated with working with unculturable pathogens have severely restricted the development of genetic and biochemical systems for studying these pathogens. Thus, genomic sequences often provide one of the first "deep" examinations of the microorganisms. A general theme that has emerged from these analyses is that many of the fastidious, difficult-to-culture pathogens are characterized by reduced genomic contents, relative to their free-living, close relatives [2,25,50,108, 154,155]. Presumably, adaptations to the intracellular environments of their hosts have rendered many of the genes of these pathogens obsolete.

An informative example of the usefulness of applying genomics technology to an unculturable microbe is that of *T. whippleii*, the causative agent of Whipple's disease (see Section 4.2.2.2 for details). Isolation of *T. whippleii* in sufficient quantities from tissue culture allowed its genome to be completely sequenced, even though axenic cultures had yet to be obtained [11]. Through subsequent analysis of the genomic sequence, Renesto et al. [139] discovered several possible deficiencies in amino acid synthetic pathways (histidine, tryptophan, leucine, arginine, proline, lysine, methionine, cysteine, and asparagine pathways were entirely missing). A growth medium designed to compensate for these deficiencies was used to propagate several independent isolates of *T. whippleii* in axenic culture, a *tour de force* that will surely inspire attempts to culture other fastidious microorganisms [139]. The study of Whipple's disease has thus profited twice from culture-independent analyses, first in the identification of the causative agent by rRNA molecular phylogenetics and second by the isolation of this organism based on insights from genomic analysis.

4.3.2 Metagenomics

Analogous to rRNA analyses of complex microbial communities, studies of mixtures of microbial genomes also have been carried out in a culture-independent manner. Dubbed "metagenomic" analysis, such studies entail the cloning and high-throughput DNA sequencing of hundreds of thousands of microbial genes. Based on similarity matches to sequences in databases, the identities of the sequenced genes from the microbial community being studied are inferred. Physiological function and metabolic lifestyles are then inferred from the putative gene identities. Collectively, this genetic information (along with rRNA analyses) provides a detailed view of the types of organisms present in a sample and the ways in which they have adapted to their environment (e.g., through symbiosis, pathogenesis, etc.). In the medical context, such information can illuminate the mechanisms by which infections develop and persist, guide the development of diagnostic resources, and suggest management schemes. Ultimately, correlation of microbial metagenomics with host gene expression data will lead to individualized prediction of disease progression and treatment outcomes.

Metagenomic studies initially were developed in laboratories studying marine microbiology [7,8,112] and rapidly expanded to other environmental locales, such as soil [145] and biofilms associated with acid-mine drainage [180]. In addition to addressing basic questions of microbial ecology, a major stimulus for pursuing metagenomic studies has been the search for novel biocatalysts (*cf.* [26,59,168]). Indeed, one of the first published metagenomics studies [7] reported the discovery of a novel light-harvesting protein, proteorhodopsin, that likely plays a major role in the primary productivity of marine bacteria [147].

The utility of metagenomics studies has not gone unnoticed by the clinical microbiology community, although few such projects have been reported to date. A particular difficulty is that the bodily sites where microbes are greatly enriched relative to host cells (thereby simplifying purification of bacterial DNA) also are home to some of the most complex microbial populations. Surveys of rRNA sequences reveal the staggering number of microbial species in the oral cavity and gastrointestinal tract. Nonetheless, several groups are conducting metagenomic analyses of these locales. The National Institutes of Health (United States) have funded a program with a goal of delineating the human oral microbiome, the results of which are eagerly anticipated. Manichanh et al. [103] have recently published a metagenomic-based comparative study of Crohn's disease that surveyed the frequencies with which different species of rRNA genes were cloned from fecal samples of patients and controls. The authors observed a significant reduction in the occurrence of members of the bacterial division Firmicutes (low G–C Gram positive) in Crohn's subjects, and concluded that this could be a signature for the disease.

Lastly, because it is not rRNA based, the metagenomic approach can be applied to viral populations as well as to cellular organisms. Breitbart et al. [14], for instance, constructed a metagenomic library of bacteriophage enriched from a single human stool sample. A tremendous degree of sequence diversity was revealed in this library, indicating the presence

of multiple types of phage; only 41% of the 532 sequences examined were significantly similar to previously reported sequences [14]. This and other studies have hinted that as the viral world becomes the subject of further culture-independent analyses, we will discover that it surpasses the complexity of the bacterial world.

4.4 FUTURE PERSPECTIVES

Standard plating techniques are now well established to underestimate the true extent and diversity of the natural microbial world [61,124,156,192]. However, the rise of molecular biology and its application to microbial phylogeny has revolutionized the microbiologist's ability to detect, characterize, and objectively identify microorganisms. This technology now permits comprehensive analyses of complex microbial communities, even in the absence of cultivation. In the near future, clinical treatment of infectious diseases will remain dependent on cultivation of pathogens, for instance when antibiotic susceptibility testing is required. However, the maturation of culture-independent technologies such as metagenomics will continue to greatly expand our understanding of the normal human microbiota and the molecular basis of pathogenicity. This knowledge will further propel the development of more rapid and informative molecular assays to detect pathogens as well as changes in the commensal microbiota. As technological barriers are broken and economies of scale are applied to mass sequencing efforts, we foresee the day when monitoring a person's microbiota becomes a common component of the health checkup. What was impossible yesterday will be routine tomorrow.

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CHAPTER 5

Molecular or Immunological Tools for Efficient Control of Tuberculosis

J.L. Herrmann and P.H. Lagrange

Service de Microbiologie, Hôpital Saint-Louis, 1 avenue Claude Vellefaux 75010, Paris, France

5.1 INTRODUCTION

Tuberculosis, the disease process caused by *Mycobacterium tuberculosis* (the Koch bacillus), is currently the second leading cause of death from an infection after HIV [39]. Both of these infectious diseases work synergistically, increasing the prevalence of tuberculosis in the developing world essentially; increasing also the risk of death in coinfected patients. Control of infectious diseases is primarily linked to two related actions: the efficient detection of active cases and efficient treatment and follow-up of treated patients to prevent the transmission. These dogmas are true for the majority of infectious diseases but remain difficult to apply to tuberculosis. Infected persons have a 10% lifetime risk of developing active disease, therefore tuberculosis is characterized by a huge human reservoir of latently infected but asymptomatic individuals (one-third of the world's population) at risk of further developing disease and transmitting the bacillus.

New insights in the tubercle bacillus behavior and in the host immune response have recently been obtained, largely due to the developments of new molecular and immunological tools. The successive discoveries of several repeated elements present on the *M. tuberculosis* chromosome have allowed the development of genotyping methods and databases that contributed to our understanding of the organism and its transmission [76]. Confirmation of deletions of genomic regions, first observed by Mahairas et al. [47], opened a tremendous era of discoveries including links between clinical strains and host geographical origin [31], the drawing of a complete evolutionary tree of all species and isolates belonging to the tuberculosis complex [13], and new families of specific antigens with a strong potential as diagnostic tool [5]. However, those molecular approaches require cultured mycobacteria. Whether there is still a future for these techniques in the study of tuberculosis epidemiology and control is presently challenged by existing immunological tools.

5.2 DEFINITIONS: CLINICAL CHARACTERISTICS OF TUBERCULOSIS

Tuberculosis is an airborne disease that is transmitted by small droplets expectorated by infectious patients. Patient infectivity is defined by the presence or absence of acid-fast bacilli (AFB) in the patient's sputum, as detected by microscopic examination of sputum smear stained by Ziehl–Neelsen or Auramine dyes (Fig. 5.1). The higher the number of visible bacilli, the more infectious the patient. Airborne transmission of *M. tuberculosis* is promoted by increasing duration and proximity of contact with an infectious case. A key determinant of infection is the amount of time spent sharing room air with the source case.

Disease expression might occur in any organ. In 70% of cases, the lungs and hilar lymph nodes are the major organ concerned, classified as pulmonary tuberculosis. Presence of the bacilli in any other organs is defined as extra-pulmonary tuberculosis. The major characteristic of tuberculosis infection is that the vast majority of persons in close contact with an infectious index case do not develop active disease. Of those infected, only 5% will develop active disease during the

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

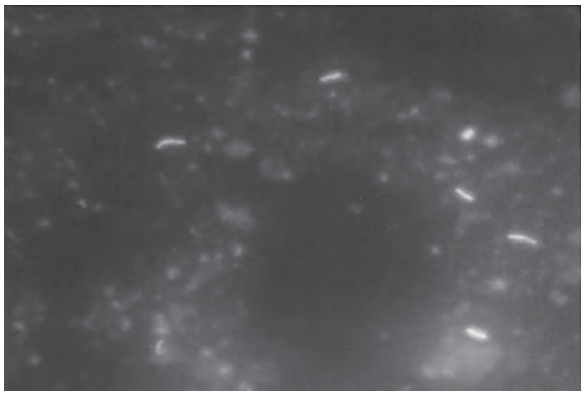

Fig. 5.1. Auramine staining of a sputum smear: we can notice the presence of acid fast bacilli, fluorescent, on a red background of eukaryotic cells. See color plates.

first 2 years following contact. A further 5% will develop the disease in the ensuing years, leaving 90% individuals considered as resistant to the infection (Fig. 5.2) [38].

Host factors play a major role in determining the risk of developing tuberculosis: persons with immunodeficiency states, resulting from coexisting diseases (such as HIV infection) or malnutrition, or infants, or those at the extremes of age, are at higher risk of becoming infected with and developing tuberculosis. Rate per year among persons infected with HIV varies from 2% to 8% [38]. Two states can therefore be defined: tuberculosis infection, where the infection remains latent, and active tuberculosis when disease is expressed and may cause progressive pathological damage unless diagnosed and treated. Latent infection can give way to active disease, when a decrease in the absolute number of CD4 cells allows the tubercle bacilli to reactivate and declare the disease (as may occur in an HIV-infected population). Animal models had previously shown that, despite an appropriate immune response or with correct treatment, infected animals were unable to eradicate the bacillus [30, and references therein].

The clinical mycobacteriology laboratory plays a key role in the control of the spread of active tuberculosis, through timely detection, isolation, identification, and drug susceptibility testing of *M. tuberculosis* isolates. However, detection rates may be as low as 20% in certain countries, and even with the best facilities available, around 25–40% in child tuberculosis, 50% in extrapulmonary tuberculosis, and 60–70% may prove culture positive, leaving in total approximately 30% of unconfirmed cases. Delays in detection, due to the slow growth of *M. tuberculosis* can hamper the efficiency of the microbiological diagnostic process. Implementation of molecular tools does not improve rapid diagnosis of tuberculosis, leaving WHO to recommend smear examination, and treatment of any AFB-positive patient. Although rapid, a sputum AFB smear has a reported sensitivity range of 22–78%. Its specificity is compromised when specimens are obtained from individuals with chronic pulmonary disease and heavily colonized with non-tuberculosis mycobacteria (NTM). In addition, patients smear negative for *M. tuberculosis* are known to transmit disease. In conclusion, multiple diagnostic tools do exist, but do not allow a rapid diagnostic performance allowing for efficient control of tuberculosis with direct benefits to public health.

The role of clinical mycobacteriology laboratories is far more limited for the diagnosis of latent tuberculosis infection. The diagnostic tool, only available until recently for latent tuberculosis infection, was the tuberculin skin test (TST) [32,44]. The development of IFN-γ-based assays recently challenged this assay [5,52]. No gold standard diagnostic test for latent tuberculosis exists, even if the TST might be considered as a reference method against which any new assays might be compared. As we will see throughout this chapter, there are two major questions when studying the epidemiology of tuberculosis and its control: (i) How can we

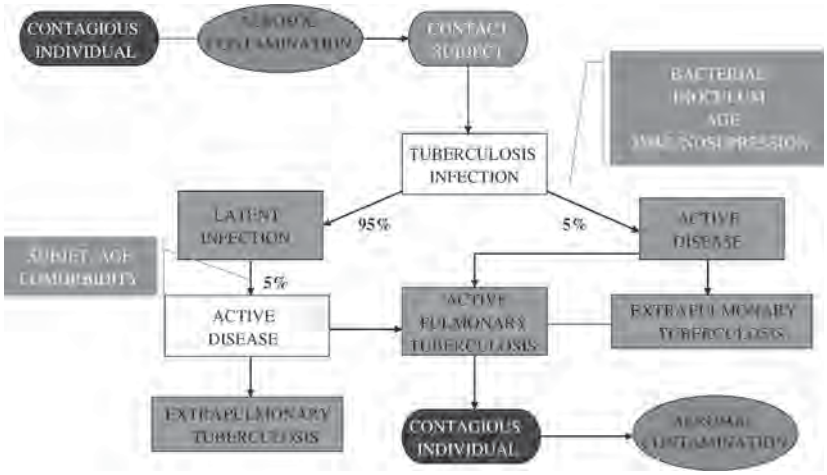

Fig. 5.2. Schematic representation of the evolution of tuberculosis after a contact with an infectious patient. The majority of infected patients are somehow protected as only 10% of them will declare the disease during their lifetime.

correctly identify infected individuals and (ii) How do we detect those, inside this group, who will go on to develop active tuberculosis?

5.3 MOLECULAR EPIDEMIOLOGY: ADVANTAGES AND DRAWBACKS

Do we have a correct idea of tuberculosis as a disease in its entirety by looking only at clinical isolates or references strains, defining their clonality or their belonging to a cluster of transmission, or by looking at individuals, that is, those infected, their detection, and prevention of further development into an active disease? Molecular tools have existed now for more than 20 years, defining what we call "*modern genotyping*." The relevance of these methods to the controlling and understanding of the pathogenesis of tuberculosis has been recently developed in several excellent reviews [76]. Briefly, these methods relied on the detection of different genetic elements, such as insertion sequences (IS*6110*) (Fig. 5.3a) [23,51], direct repeat (DR locus) (Fig. 5.3b) [29], variable number of tandem repeats (VNTR) [26,68], polymorphic G–C-rich sequences [56], and single-nucleotide polymorphisms [1].

Efficient tuberculosis control is best demonstrated by a reduction in case rate, higher treatment completion rates, low transmission rates, and more effective case finding and treatment of infected contact, underpinned by a multifactorial nature of each measure [10]. However, the efficiency of molecular methods in terms of public health benefits is still limited. These methods require weeks or months to complete, and patients are usually well into a course of treatment before fingerprint evidence of a false-positive culture is available. Implementation of molecular typing based on PCR, such as

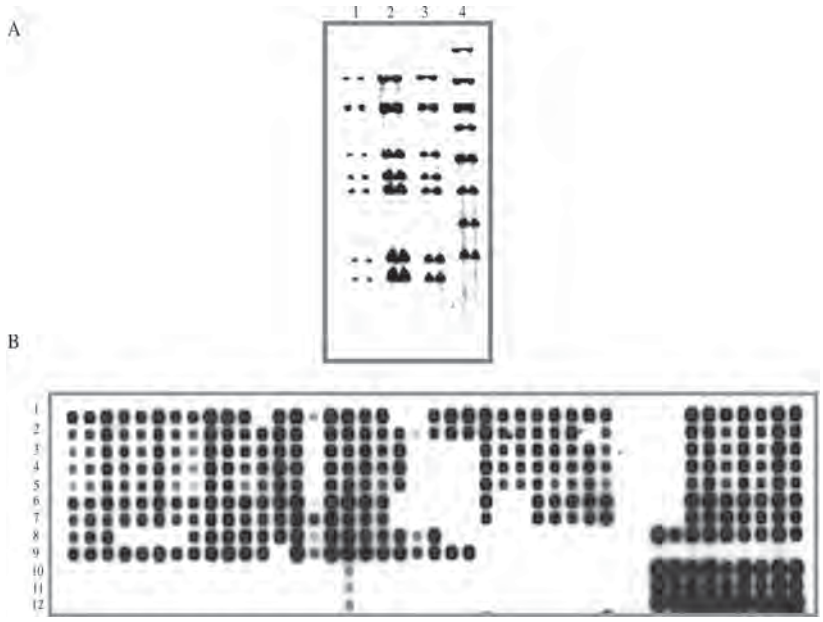

Fig. 5.3. Examples of Southern blot, representative of molecular tools used in tuberculosis epidemiology: (a) represents the IS*6110* restriction fragment length polymorphism called IS*6110* RFLP. Briefly, IS*6110* is a repetitive element (or an insertion sequence) presents between one and more than 20 copies on *M. tuberculosis* genome. After DNA extraction from *M. tuberculosis* clinical isolates, and restriction by PvuII, restricted DNA is then loaded on an agarose gel. After migration, a transfer on a nitrocellulose membrane is performed, and DNA is then hybridized to specific 5′ probe of the IS*6110* element. Homologous strains have identical IS*6110* RFLP as shown for the first three lanes, and different isolates have different IS*6110* RFLP, the fourth lane is representative of a different isolate as compared to the three others (Herrmann and Lagrange, unpublished results). (b) represents the detection of DR (for direct repeat) polymorphism by the technique called spoligotyping. The DR region comprised repetitive elements (DR) and interspersed DNA sequences. By performing a multiplex PCR directly from clinical isolates or from purified DNA, size-different biotynilated amplicons of this region are then hybridized on a pre-prepared nitrocellulose membrane. The membrane has already fixed all the oligonucleotides specific of the interspersed DNA sequences. The presence of a DNA sequence is noted by a black signal, and its absence by an absence of signal. This notified the presence or the absence of a DR. Certain spoligotyping profiles, called spoligotype, are specific of a clinical isolate found throughout the world, in our example the three last right lanes are characteristic of Beijing isolates (Herrmann and Lagrange, unpublished results).

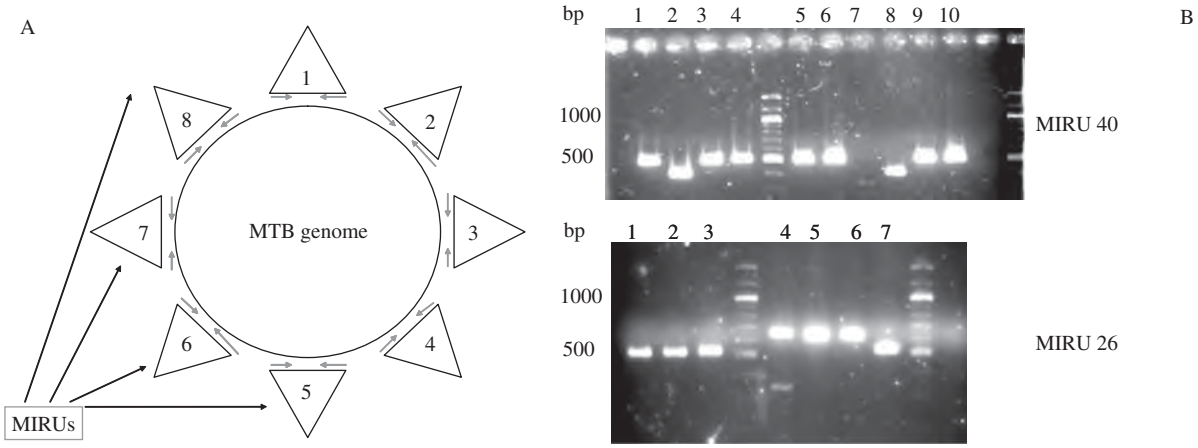

Fig. 5.4. (A) Schematic representation of the dispersion of mycobacterial interspersed repetitive units or MIRUs. MIRUs are simply repetitive sequences present at different loci, and of size normally of 77 bp, although several might be shorter (51 bp). Twelve MIRUs are normally sufficient for the differentiation of *M. tuberculosis* isolates as compared to IS*6110* RFLP. As shown in (B) each clinical laboratory can perform a simple PCR reaction using first the most polymorphic of the 12 MIRUS, like MIRU 40 or MIRU 26. Differences between clinical isolates are easily visualized by the size difference of the amplicons (lanes 2, 7, and 8 for MIRU 40 or lane 7 for MIRU 26). A single difference, that is, one MIRU with a different size, is sufficient to differentiate the isolates. A more efficient way to apply MIRUs is to perform three times four different Multiplex PCRs (12 MIRUs) and to apply the labeled amplicons on an automatic capillary sequencer (see for details Supply et al., and its dedicated website).

spoligotyping (Fig. 5.3b) [36] and the mycobacterial interspersed repetitive units (MIRUs) (Fig. 5.4a and b) [49], will allow a considerable reduction in the time between isolation and comparison of several isolates to confirm their identity.

The major contribution of DNA fingerprinting through these methods is the ability to highlight previously unsuspected transmission in the community and areas in which contact tracing is not working [61], identifying laboratory cross-contamination, and differentiating recurrent tuberculosis caused by treatment failure or relapse from exogenous reinfection; techniques therefore are mainly applicable in low-incidence countries.

Molecular epidemiological studies are usually performed on a small scale, as it is not usually possible to allow complete collection of strains. Consequently, these studies have to be interpreted with great caution [10]. One of the major debates relates to the proportion of clustered cases in low- or high-incidence countries. This percentage in Europe varies from 16% to 46%, suggesting that recent transmission of tuberculosis can be an important factor even in low-endemic areas. However, as demonstrated previously [76], implementation of DNA fingerprinting progressively over 2 years has increased the percentage of clustered isolates sharply. Thereafter, the increase of the clustering percentage was almost negligible. This suggests that the extent to which clustering reflects recent transmission depends strongly on study duration. In addition, small studies in high-incidence areas will severely underestimate recent transmission. Similarly, sampling will bias the estimate of clustered cases and the effect of risk factors for clustering.

Even if molecular typing methods can identify settings for transmission (households, family members, and high-risk groups), they still rely on documented cases, that is, culture positive tuberculosis cases. These methods showed however that several isolates were more transmissible and associated with tuberculosis outbreaks (strain W, Beijing, 210) (Fig. 5.3b) [11,73,75] but confounding perhaps the issue of transmissibility and virulence. Only recent approaches have clearly demonstrated a peculiar evolution of *M. tuberculosis* isolates [70]. First, comparison of the *M. tuberculosis* H37Rv genome with 100 clinical isolates using high-density oligonucleotide array [31,71] identified deletions throughout the genome, with deleted genes representing ancestral genes no longer needed for strain survival [71]. Large deletions are assumed to play a major role in the molecular evolution of *M. tuberculosis*. The proposal is that isolates harboring those deletions have a short-term relative advantage. Correlatively, the usage of deletion as a phylogenetic marker allowed the demonstration that *M. tuberculosis* clinical isolates that infected patients in their mother countries, and even migrants developing overt disease in San Francisco tend to be infected by those strains that are more specific for their regions of origin [31]. These studies confirm what has been known for a long time, that transmission of tuberculosis requires extensive contact, impacting sociological parameters. So in addition to the MIRUs studies, which demonstrated the clonality of *M. tuberculosis* isolates [69], we now have the existence of an intimate link between the strain and its host [70].

In conclusion, molecular tools have permitted a better knowledge of *M. tuberculosis*, confirming the different social risk factors for being infected with *M. tuberculosis*, but still

lack the ability to provide an overview of the disease itself related to the host.

5.4 IMMUNOLOGICAL EPIDEMIOLOGY

5.4.1 The Immune Response in the Control of Tuberculosis

To estimate the burden of recently transmitted tuberculosis, we need more than contact tracing and molecular approaches. Recent advances in the knowledge of the genome of *M. tuberculosis* [17] have allowed an era of genomic comparison using micro- or macro-arrays and the discovery of deletions (see above) (Fig. 5.5). Several genomic regions present in *M. tuberculosis* and/or *M. bovis* are absent from the vaccine strain *M. bovis* BCG [13,28,47]. One of this region, region of deletion 1 (RD1) (Fig. 5.5), code for a family of small secreted proteins named ESAT-6, and others such as CFP-10 [4].

Although RD1 is absent from most environmental mycobacteria, the genes coding for ESAT-6 and CFP-10 were demonstrated to be present in *M. kansasii*, *M. marinum*, *M. szulgai*, *M. flavescens*, *M. gastrii*, and *M. leprae* [8]. This raises the question of whether infection with or even exposure to *M. kansasii* or *M. marinum* can induce T-cell responses to ESAT-6 and CFP-10, as recently demonstrated [8]. These results have to be taken into consideration regarding the development and the use of ESAT-6 and CFP-10 as target antigens in tuberculosis patients. However, tuberculosis is far more frequent as compared to mycobacteriosis due to *M. kansasii* or *M. marinum* in undeveloped countries. These infections are also rarely observed in developed countries, mainly in immunocompromised patients and in patients with underlying pulmonary diseases such as lung cancer, bronchectiasis for *M. kansasii*, or in patients with professional risks such as fishmonger, or owners of aquarium for *M. marinum*.

The major advantage of immunological assays in the diagnosis of infectious disease is their ability to study the host response without the need for bacterial isolation. We know the limitations of classical microbiological techniques. Not enough has been done until now in the development of an immunological approach, mainly due to the lack of sensitivity and specificity of antigens used.

After infection takes place, innate immunity plays a major role in the control of further spread of the tubercle bacilli. Alveolar macrophages are the first point of contact with the bacteria. They are permissive and allow bacterial replication [60]. Phagocytosis of mycobacteria or the apoptotic bodies generated by dendritic cells present in the lung parenchyma allow the maturation of these cells, their migration to the draining lymph mode and activation of naive T lymphocytes. These cell-to-cell interactions lead to the production of an adaptive immune response, with the production of IFN-γ essential for protective immunity against *M. tuberculosis* [38].

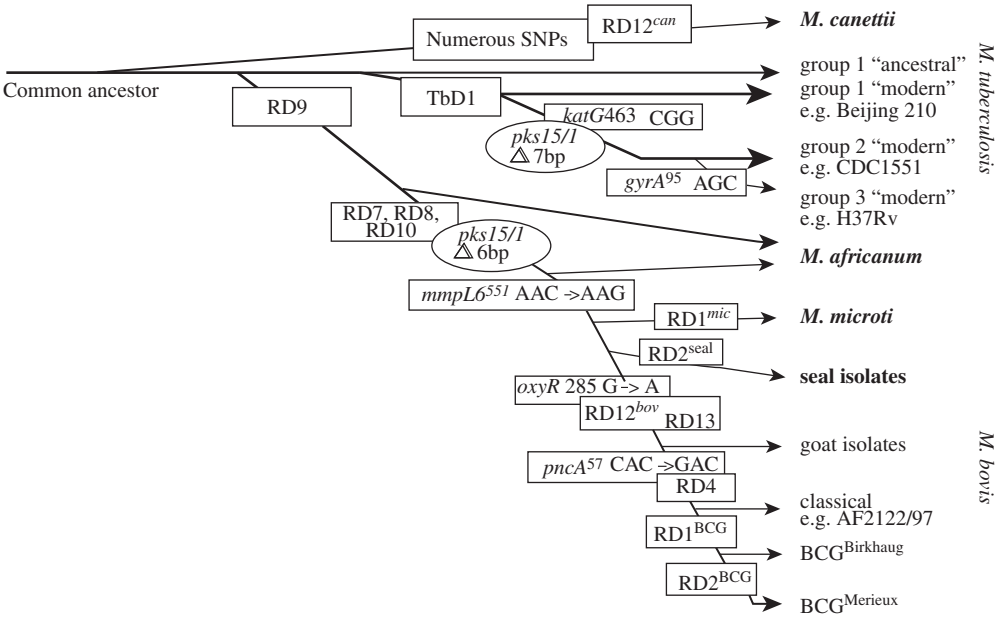

Fig. 5.5. Scheme of the proposed evolutionary pathway of the tubercle bacilli kindly provided by Roland Brosch (Unité de Génétique Moléculaire Bactérienne, Institut Pasteur, Paris, France) illustrating successive loss of DNA in certain lineages. The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes. Of interest, is the characterisation of deleted regions absent from *M. bovis* BCG and present in *M. tuberculosis* or in *M. bovis*. These regions code for proteins that can be used as target antigens for new assays like the assays described in the text.

This cellular response circumvents bacterial replication inside a granuloma. However, as shown by historical inoculation studies in small animals and recently described by Hernandez-Pando et al. [30], the tubercle bacillus is not eradicated, and can persist in the lungs, mainly in noninfected areas of the lungs in the periphery of the granulomas and also in lung epithelial cells [30]. During this entire process, antibody production is absent or undetectable, and the only way to characterize an infected patient is to rely on assays evaluating the cell-mediated-immune response such as the well-known TST or the newly described IFN-γ-based assays. Whether these IFN-γ-based assays will allow an improved knowledge of tuberculosis epidemiology is presently under scrutiny.

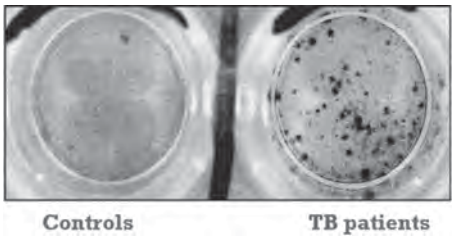

Fig. 5.6. Example of an ELISPOT result. ELISPOT technique relies on the separation of B or T lymphocytes which are then loaded on a nitrocellulose membrane and a specific antigen is added. Antibodies producing cells for B cells or IFNγ producing cells for T cells, are detected by monoclonal antibodies against human IgG or IFNγ. A positive cell is visualized by microscopy as a spot (here in black). The number of spots gave the number of positive B or T cells.

5.4.2 IFN-γ-Based Assays: Description—Gold Standard of Tuberculosis Infection

The TST was first introduced in 1890 and used for decades; it was the only test to diagnose latent tuberculosis [32,44]. However, the antigen used, protein purified derivative or PPD, is a crude mixture of more than 200 antigens, many of which are shared among *M. tuberculosis*, *M. bovis* BCG, and several NTM. As a result, TST has a lower specificity on populations with high BCG coverage and NTM exposure. It also presents several operational drawbacks including the need for a return visit and operator-dependant variability in placement and reading of the test.

The immune response against *M. tuberculosis* is highly dependent on IFN-γ production by antigen-specific thymodependent lymphocytes (T cells). Over the past decade, there has been an increasing interest in the development and application of an *in vitro* culture assay measuring IFN-γ production in response to stimulation by mycobacterial antigens as a substitute diagnostic screening test for the classical TST [45]. IFN-γ-based assays detect the presence of T lymphocytes in each individual responding to specific *M. tuberculosis* antigens. By collecting peripheral blood by venopuncture, peripheral blood mononuclear cells (PBMC) are incubated either directly or after Ficoll purification with two or more *M. tuberculosis* antigens. A positive response is obtained when IFN-γ, synthesized by activated T cells, is detected either by an ELISPOT (Fig. 5.6) or an ELISA approach. The T cells detected are either effector T cells or memory T cells and the contact of the PBMC with the antigens varies from 24 h to 5–6 days. Initially using purified PBMC, the methodology evolved to a whole blood culture technique that was first validated in Australian cattle [59]. These studies demonstrated that the IFN-γ assay had greater diagnostic sensitivity, lower cost, and rapid results for cattle TB screening and was further developed for human testing initially using human PPD, avian PPD, and the mitogen phytohemagglutinin as a positive control.

Evaluation of these assays in comparison with the classical TST has demonstrated agreement ranges from 40% to 100% in latent tuberculosis infection or in active tuberculosis, the lowest agreement being obtained in active tuberculosis. Higher agreement was always correlated with recent exposure [52, and references therein]. However, as observed with TST, using the PPD antigens renders the assay nonspecific due to the cross-reactivity between PPD and other mycobacterial species. Stretton et al. [67] included subjects being BCG vaccinated in their study and were unable to discern the effect of BCG on the assay results.

Since the beginning of the year 2000, two antigens, ESAT-6 and CFP-10 encoded by RD1 absent from the vaccine strains [13,28], demonstrated clearly their superiority in evaluating T-cell response in patients with active tuberculosis as compared to TST, with a very high specificity (93%) [5]. Percentages of patients with TB disease who responded to ESAT-6 (or CFP-10) are between 60% and 80% in low-endemic countries such as Denmark, United States, Germany, and Kuwait. By comparison, T-cell responses were constantly negative for patients with *M. avium-intracellulare complex* infection [74], those recently vaccinated with the BCG [34] or in noninfected or nonexposed people [6,55,58]. Its major diagnostic value was demonstrated in case–contact study in low-endemic countries [25,41]. However, its role in the diagnosis of latent tuberculosis infection has suffered from the lack of a gold standard, and definitions of a gold standard also may differ between studies. As mentioned by Pai et al. [52], a direct estimation of sensitivity and specificity for latent tuberculosis infection is not applicable, and the use of TST as one component of the gold standard is still an approach fraught with problems. But, studies performed during a community tuberculosis outbreak using a whole blood or an ELISPOT format demonstrated the excellent correlation between IFN-γ-based assay (detecting tuberculosis infection) and exposure to tuberculosis by using proximity, and duration of exposition with the index case [12,42]. The results were never correlated or associated with BCG vaccination, demonstrating its precision for the diagnosis of tuberculosis infection compared to the classical TST [34,42]. A high specificity (98.1%) and sensitivity (89%) of the whole blood format were observed in people with no identifiable risk for *M. tuberculosis* exposure and in patients with documented

tuberculosis, respectively [50]. Lower sensitivity values were observed in severe forms of TB disease or in disseminated TB (our unpublished results).

By comparison, in highly endemic countries for tuberculosis, positive responses were obtained in healthy contacts. Recent examples in India or in the Gambia [43,77] tend to demonstrate that IFN-γ-based results reflect the prevalance of the infection in the population. Nearly 69–80% of controls, classified as healthy controls, were positive by these assays. In addition, a strong recognition of ESAT-6 was found to correlate with the subsequent development of active tuberculosis, in contrast with PPD where similar responses were observed regardless of clinical outcome [21]. This study performed in Ethiopia opens the door for the routine use of selective treatment given to high responders, to reduce the risk of further development of active tuberculosis. It is known that chemoprophylaxis of recently infected individuals prevents the development of active tuberculosis [2]. A 300 mg daily dose of Isoniazid reduces the risk by 65% after 6 months treatment and 75% after 9 months, showing that preventive therapy can be highly cost-effective.

However, several limitations exist for IFN-γ assays, differentiation between active and latent tuberculosis, and dating of the infection being the two prime examples. Supportive clinical evidence will make the difference in the first case, or detection of replicating mycobacteria in the host [66] (and see below). In the second case, no diagnostic tools are currently capable of dating the primary infection. Considering their specificity, IFN-γ-based assays may represent the first available assays in case–contact studies able to specifically date the infection after contact with an infectious patient, and might allow clinicians to monitor those infected individuals to determine the duration of the IFN-γ response or to follow the evolution into active disease. These studies, although difficult to perform, will determine the potential and the accuracy of IFN-γ-based assay in establishing the exact proportion of infected individuals in the population.

Finally, knowledge about the performance of these assays in immunocompromised individuals, in children, and in high-risk populations in endemic countries is presently limited. Contradictory studies in tuberculosis patients coinfected with HIV have been published recently [16,24]. There have been no studies in children performed on a large scale, except recent case–contact studies, and we all know that children subject to recent transmission are more inclined to declare active disease as compared to adults.

5.4.3 Impact of T-Cell or B-Cell Assays in the Diagnosis of Active Tuberculosis

5.4.3.1 Circulating T-Lymphocytes and tuberculosis As cited previously, the quantitative response of IFN-γ might be associated with an increased likelihood of progression from tuberculosis infection to active disease: those who produced the highest IFN-γ response were at the highest risk of developing tuberculosis in the next 18 months [21].

Treatment efficacy in patient with active tuberculosis is usually monitored by evaluating early clinical and delayed radiological findings, backed up with bacteriological data. Very few studies have been published in relation to the evolution of immune parameters during antituberculosis treatment follow up.

Results of studies examining the dynamics of the T-cell responses (using the IFN-γ assays) in active tuberculosis undergoing antituberculosis chemotherapy are apparently conflicting, with either a reduction [41,54], an increase [7,72] with a persistence up to 17 years [80], or a steady state [58]. Such variability might be linked to the operational characteristics of IFN-γ-based assays used in each study, i.e., incubation period, whole blood versus purified PBMC, the use of PPD or RD1 antigens. Short incubation period (16–24 h) such as described in the whole blood assay (QuantiFeron-TB Gold, Cellestis, Australia) or in the ELISPOT technique (Oxford, UK) might detect short lived-effectors T-cells. Long incubation times (up to 5–6 days), such as those used in whole blood or PBMC in culture with specific antigens, might detect a mixture of specific effectors and memory T cells [35]. Short-term exposure to antigens will more likely evaluate the T cells associated with recently encountered mycobacterial antigens *in vivo*, which can then rapidly release IFN-γ when reexposed to the same antigens.

This was showed recently [14] with an *in vitro* assay detecting short-term T-cell-mediated IFN-γ responses to selected ESAT-6 peptides of *M.tuberculosis* and was useful in monitoring the effectiveness of antituberculosis treatment. The decrease in specific T-cell numbers has been measured at only two time points: after 3 months and also after the 6 months time in the responders and nonresponders.

5.4.3.2 Circulating B-lymphocytes and active tuberculosis ELISPOT assays detecting the presence of specific circulating antibody secreting B cells have been developed for several infectious diseases [9,57,66]. These studies have demonstrated a correlation between the detection of specific circulating B cells and the presence of antigens secreted by replicating microorganisms. Therefore, the use of ELISPOT assays may be informative in the analysis of chronic infectious diseases either to make a diagnosis of reactivation or to monitor treatment effectiveness. The dynamics of the specific antibody-secreting B lymphocytes in tuberculosis patients has been described recently [66]. This assay measures the number of spots corresponding to the circulating antibody secreting cells (ASC) detected by antigens loaded onto an *in vitro* cellulose membrane, counting circulating specific B lymphocytes from purified PBMC from documented tuberculosis patients and responding to the presence of mycobacterial antigens [66]. *M tuberculosis* antigens used in the assay were those released early in culture supernatant during mycobacterial growth *in vitro* or short-term-culture-filtrate-antigens (STCF-antigens) [4]. The results obtained with this assay allowed several observations to be made. Before treatment, 80% of TB patients had significantly more specific ASC compared to the controls

($p < 0.0001$). Antituberculous treatment was responsible during the first week for a rapid and significant increase in the number of ASC with a positive ELISPOT for all studied patients at day 8 (100% sensitivity). During the follow up, a constant and exponential decrease in the number of ASC was observed (with an observed rate of decay of 33% per week) followed by their disappearance at the end of the first month of treatment. The ASC decrease occurred before the conversion of smear positivity and as a consequence before the culture became negative.

In a given individual, the frequency of circulating ASC is thought to be largely driven by B cell activation induced by the antigen load [9]. This is probably why the frequency of ASC in patients with TB increased first during the early phase of the treatment and then decreased very dramatically a few days later with successful therapy [66]. Thus unlike TST and serological tests, the *ex vivo* B cell ELISPOT is dynamic and produces quantitative responses to STCF-antigens, representing as such an indirect marker of mycobacterial viability. Quantification of specific ASC might serve as an indirect measure of replicating bacterial burden that could be used to monitor the diagnosis and response to TB treatment. No other biological marker exploring the humoral immunity or cellular immunity has demonstrated a similar correlation with the disappearance of live tubercle bacilli in sputum and with a positive outcome.

These results suggest that the quantitative relationship between levels of effector T cells or B cells (as measured by ELISPOT or whole-blood assay), antigen load and bacterial burden can be exploited to monitor the response to tuberculosis treatment. In addition, it helps to differentiate active tuberculosis with viable mycobacteria from latent tuberculosis with the absence of replicating mycobacteria.

5.4.3.3 Antibodies: limit of their detection; advantages of non-proteic antigens for the diagnosis of tuberculosis The use of serology in the diagnosis of tuberculosis has a long record in the tuberculosis literature, but has never been developed due to its low diagnostic value with poor specificity and sensitivity [15,27,78]. Since the 1990s, newer approaches have been chosen, using enzyme-linked immunsorbent assays (ELISA) and highly purified protein antigens produced mainly by recombinant technologies, and improvement of these assays has been obtained by mixing several different antigens [15]. However, a much lower frequency of IgG antibody to *M. tuberculosis* protein antigens was always reported in HIV+/tuberculosis coinfected patients as compared to non-HIV/tuberculosis coinfected patients [20,79].

A panel of non-protein antigens represented by glycolipids specific for *M. tuberculosis* has been developed [18,19,53,62–65] and evaluated in different patient populations. Seventy percentage of HIV patients coinfected with *M. tuberculosis* had serum reactivity to at least one glycolipid antigen and maintained the diverse antibody repertoire previously observed in HIV-negative tuberculosis patients. The reason underlying the lack of antibodies in 25–30% of the tuberculosis patients was shown to be due to the presence of antibodies in circulating immune complexes and their detection improved the overall sensitivity without changing specificity [64]. The presence of specific antibodies to glycolipid antigens has also been reported with varying frequencies according to the antigens tested. These different sensitivities are in fact due to the heterogeneity of patient responses. Some patients responded to only one antigen, some against two antigens, and others to all three antigens as previously reported [46,62], and an improvement in the sensitivity is demonstrated by combining the results obtained for the three glycolipid antigens. By comparison, the sensitivity of testing for antiglycolipid antigens was equal among HIV-positive patients with pulmonary tuberculosis and those with paucibacillary (smear negative) and extrapulmonary tuberculosis.

The second point concerns the potential value of our ELISA test to predict the development of tuberculosis in high-risk HIV patients, since several HIV infected patients have high levels of specific antibodies several months before developing the disease [40]. Currently, PPD is used to identify persons with previous exposure to *M. tuberculosis*, and in the HIV-infected population, in view of the high risk of reactivation of latent infection, to identify those in whom preventive treatment might be beneficial. Antiglycolipid antibodies are present in HIV positive tuberculosis patients several months preceding any clinical manifestation of tuberculosis, as previously reported [3,48]. Such observations could indicate that this method might help in decision-making on the use of prophylactic antituberculosis therapy in HIV infected patients at high risk to develop tuberculosis. However, anti-DAT and anti-PGLTb1 antibody levels were shown to decline very slowly during the 6–9 months period of treatment, indicating that this method of antibody surveillance is not useful in evaluation of treatment efficacy [22,33,37,66]. Likewise, for a new patient suspected of tuberculosis, it is important to know if the patient has already been treated in the recent past for a first episode of tuberculosis, in order to validate the interpretation of the antiglycolipid antibodies results. The presence of circulating antibodies to glycolipid antigens for extended time periods prior to the development of clinical disease suggests that replication of *M. tuberculosis* is a dynamic process *in vivo,* which occurs prior to the deterioration of cellular immunity sufficient to allow clinical disease to develop. Their individual kinetics did not yield any information towards early treatment outcomes. These data confirm the lack of predictive power of serological tests in facilitating treatment monitoring.

5.5 CONCLUSIONS

Immunological approaches for the control of tuberculosis and its epidemiology, including diagnosis of latent or active tuberculosis and treatment follow-up, are at present extremely

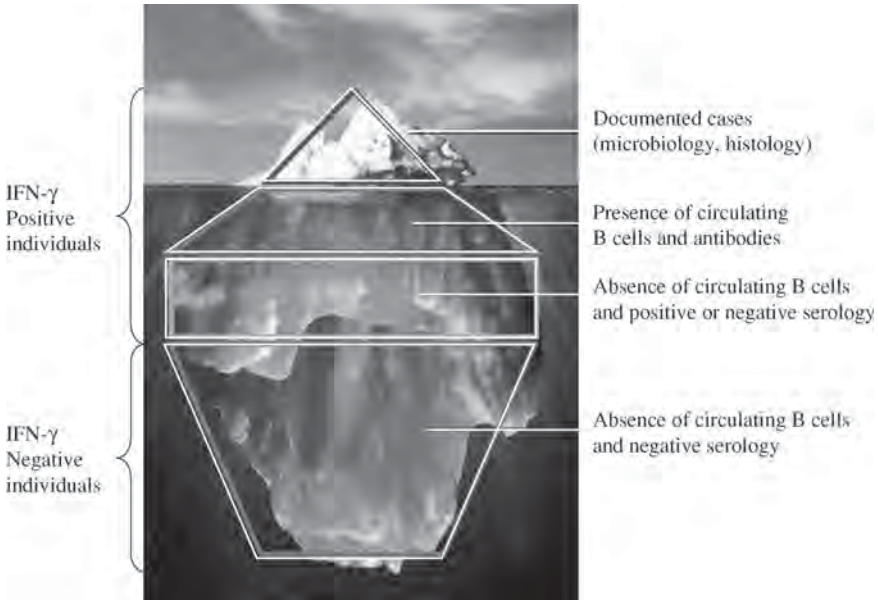

Fig. 5.7. Tuberculosis population might be compared to an iceberg, with a vast majority of unsuspected threat. Immunological assays, as developed in several laboratories, might represent the clues by which this threat might be uncovered. The top population represents the documented cases, with clinical isolates used for the molecular epidemiology. Knowing the lack of exhaustivity of strains collection in epidemiological studies, this scheme demonstrates the difficulty of dealing only with documented cases for a complete picture of tuberculosis epidemiology. Evaluation of the immune responses in individuals will allow to be more specific in the definition of infected individuals, and in their follow-up as immune markers might now tell us if the bacteria inside the host replicates or not, which in this case is indicative of chemoprophylaxis in the absence of clinical symptoms or treatment in the case of clinical or subclinical signs of tuberculosis.

promising. IFN-γ-based assays will determine with high specificity the exact proportion of *M. tuberculosis* infected individuals (Fig. 5.7). Despite the fact that the date of initial infection cannot be determined, it will still allow the calculation of prevalance of the infection in the population. Whether or not this methodology will influence the decision to give chemoprophylaxis to the infected people is dependant on political decisions in term of health-care, but it is important to remember the cost-effectiveness of such a practice. Additionally, detection of circulating B-lymphocytes in patients, will detect those developing active tuberculosis, as the presence of specific antibodies secreting cells has always been correlated with the presence of replicating bacteria. This is the main difference with detection of circulating antibodies, for which a sustained level is observed independent of treatment success.

With a better understanding of the infected population, in case–contact studies, judicious use of both molecular and immunological methods will help improve our understanding of the propensity of a mycobacterial isolate to be transmitted and its ability to induce disease in the infected host. It will also allow detailed follow up of infected people to determine the host risk-factors resulting in the development of active tuberculosis.

ABBREVIATIONS

AFB:	Acid-fast bacilli, one of the main characteristic of mycobacteria is to resist to the distaining effect of acid and alcohol as compared to other bacteria.
ASC:	Antibody secreting cells
BCG:	Vaccine strain called bacille of Calmette and Guerin
CFP-10:	Culture filtrate protein 10kDa
DAT:	Di-acyl trehalose
ELISA:	Enzyme-Linked ImmunSorbent Assays
ESAT-6:	Early secreted antigen tuberculosis of 6 kDa, and T-cell reactive
HIV:	Human immunodeficiency virus
IFN-γ:	Interferon gamma
MIRU:	Mycobacterial interspersed repetitive units
NTM:	Non-tuberculous mycobacteria, which represents mycobacteria which did not belong to the tuberculosis complex
PBMC:	Peripheral blood mononuclear cells
PCR:	Polymerase chain reaction
PGLTb-1:	Phenolglycolipid tuberculosis antigen 1
PPD:	Purified protein derivative (crude extract of mycobacterial antigens)
STCF:	Short term culture filtrate

TST: Tuberculin skin test, which represents the ancient way of detecting individuals after a tuberculosis contact, or vaccination by the BCG strain

GLOSSARY

CD4 cells: Represents T lymphocytes, expressing the surface receptor CD4.

Direct repeat: The mycobacterial DNA, as many other DNAs possess several similar DNA sequences that are repeated throughout their genome.

Insertion sequence: An insertion sequence is a small piece of DNA, flanked by two direct repeats, able to jump on the same DNA, or to be transmitted into another bacterium by conjugation, or natural transformation.

Polymorphic G-C rich sequences: *M. tuberculosis* has a high GC content genome. This property has been used to labelled Southern blot of mycobacterial DNA with rich GC probes, giving a picture similar to IS*6110* RFLP, although with more bands. This, in fact, renders the GC-RFLP more difficult to interpret.

Region of deletion: One of the main advances in tuberculosis research has been the complete sequencing of *M. tuberculosis* genome, and other mycobacterial genomes now. This has allowed a comparison between sequenced genomes allowing the discovery of region of DNA absent from one species and present in another species.

Single nucleotide polymorphisms: By sequencing genes, and comparing sequences obtained from different clinical isolates, researchers have been able to establish lineage and relationship between isolates (See Ref. 13, and references herein).

Spoligotyping: Technique develops recently, which includes a PCR and a reverse dot-blot. Specific oligonucleotides are fixed on a nitrocellulose membrane, and labelled DNA is added on this already prepared membrane (see Fig. 3).

Variable number of tandem repeat (or MIRUs): Are similar to direct repeat, although they can be represented on the chromosome at more than one copy, which explained the variable number. For one locus, one, two, three…of the same sequence can be repeated, and the number of repeats per locus is specific of an isolate, or an individual. It is similar to what is called minisatellites in eukaryotes.

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CHAPTER 6

Understanding Human Leishmaniasis: The Need for an Integrated Approach

M. Hide,[1] B. Bucheton,[1] S. Kamhawi,[2] R. Bras-Gonçalves,[3] S. Sundar,[4] J.-L. Lemesre,[3] and A.-L. Bañuls[1]

[1]*Génétique et Evolution des Maladies Infectieuses (UMR CNRS/IRD 2724), IRD de Montpellier, 911 av Agropolis BP 64501, 34394 Montpellier Cedex 5, France*

[2]*Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA*

[3]*Pathogenie des Trypanosomatidés (UR008), IRD de Montpellier, 911 av Agropolis BP 64501, 34394 Montpellier Cedex 5, France*

[4]*Infectious Diseases Research Laboratory, Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, 6 SK Gupta, Nagar, Lanka, Varanasi 221005, India*

6.1 GENERALITIES ON LEISHMANIASIS

Leishmaniasis has been known for many hundreds of years, with one of the first clinical descriptions made in 1756 by Alexander Russell and called Aleppo boil. Many names correspond to this group of diseases: kala-azar, Dum-dum fever, white leprosy, espundia, pian bois, and so on. Leishmaniases are parasitic diseases spread by the bite of the infected female phlebotomine sand fly (Fig. 6.1). Leishmaniases are caused by approximately 20 species, pathogenic for humans, belonging to the genus *Leishmania* (kinetoplastids order, Honigberg, 1963) and within 500 known phlebotomine species, of which only some 30 have been positively identified as vectors of these pathogenic species.

6.1.1 Geographic Distribution

Human leishmaniases are found on all continents, except Antarctic and Australia. However, cutaneous leishmaniasis was recently revealed in Australian red kangaroos [296]. Approximately 350 million people live in endemic areas, thereby comprising populations at risk, and annual incidence is estimated at 1–1.5 million cases of cutaneous leishmaniasis plus 500,000 cases of visceral leishmaniasis; overall prevalence is 12 million people. Most of the affected countries are in the tropics and subtropics: more than 90% of the world's cases of visceral leishmaniasis are in India, Bangladesh, Nepal, Sudan, and Brazil (Fig. 6.2), 90% of all cases of mucocutaneous leishmaniasis (Fig. 6.3) occur in Bolivia, Brazil, and Peru, whereas 90% of all cases of cutaneous leishmaniasis (Fig. 6.3) occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria (for further detail, see http://www.who.int/leishmaniasis/en/).

6.1.2 The Players in Leishmaniasis

Leishmania parasites are responsible for cutaneous forms as well as visceral forms of the disease. Healing or progression of this infection is related to the genetic and immune status of the *host*, the virulence and pathogenicity of different species and strains of *Leishmania*, and the *vector* involved. The hosts can be humans but also rodents, dogs, and other mammals [16,307], and great diversity of immune response exists depending on the host considered (see Section 6.4 for details). Similarly, within 500 known phlebotomine species, only 31 have been positively identified as vectors of the *Leishmania* pathogenic species and 43 as probable vectors [181]. Among them, some vectors such as *Phlebotomus Phlebotomus papatasi* and *P. Paraphlebotomus sergenti* can only be

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Fig. 6.1. *Phlebotomus argentipes*, the vector of kala-azar in India and neighbouring countries, engorged. (Photo taken by Edgar D. Rowton, all rights reserved.)

infected by one *Leishmania* species, whereas *Lutzomyia longipalpis* is a permissive vector, able to transmit different *Leishmania* species (see Section 6.2 for details). Finally, the 20 species described as pathogenic for humans belong to the *Leishmania* genus (Ross, 1903). They are divided into two subgenera (*Leishmania* in the Old World (Saf'Janova, 1983) and *Viannia* in the New World (Lainson and Shaw, 1987)), the *Leishmania* subgenus is composed of several species or species complexes (*Leishmania donovani* complex, *L. mexicana* complex, *L. major*, *L. tropica*, etc.) and the *Viannia* subgenus contains species of the *L. braziliensis* complex (*L. braziliensis* (Viannia, 1911), *L. peruviana* (Velez, 1913), and the *L. guyanensis* complex (*L. guyanensis* (Floch, 1954), *L. panamensis* (Lainson and Shaw, 1972)), *L. lainsoni*, etc.). These *Leishmania* species are associated with different diseases (see Section 6.3 for details). For example, infections by *Leishmania donovani* complex species are associated with visceral leishmaniasis and *L. braziliensis* infections are responsible for mucocutaneous

Fig. 6.2. Distribution of visceral leishmaniasis (WHO website: http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html).

Fig. 6.3. Distribution of cutaneous leishmaniasis (WHO website: http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html).

leishmaniasis. However, the first species complex is able to generate benign cutaneous lesions, and *L. braziliensis* has been isolated from simple cutaneous lesions but also from visceral forms. It is clear that *the clinical outcome of infection depends on a multifaceted association of factors among the three main players involved*: *hosts, parasites, and vectors*.

6.1.3 The Life Cycle of the *Leishmania* Parasite

Leishmania parasites are transmitted to their host by the bite of an infected female phlebotomine sand fly (Psychodidae family, Phlebotominae subfamily), which needs a blood meal to produce its eggs (Fig. 6.4). The sand fly vectors are primarily infected when feeding on the blood of an infected individual or a vertebrate reservoir host. Many mammal species could act as a reservoir host, for example, rodents or dogs [16,307].

During feeding, host macrophages, containing *amastigotes* (Fig. 6.5), are ingested by the vector. These parasite forms, round and nonmotile (3–7 μm in diameter), are released into the posterior abdominal midgut of the insect, where they transform into *promastigotes* to begin their extracellular life cycle in the vector. This form is motile, elongated (10–20 μm), and flagellated (Fig. 6.6).

The promastigotes then migrate to the anterior part of the alimentary tract of the sand fly where they multiply by binary fission. Approximately 7 days after feeding, the promastigotes undergo metacyclogenesis and become infectious (metacyclic promastigotes). They are released into the host together with saliva when the sand fly lacerates the skin with its proboscis during feeding. The sand flies usually feed at night while the host is asleep.

These metacyclic promastigotes are taken up by host macrophage, where they metamorphose into the amastigote form. They increase in number by binary fission within the phagolysosome until the cell eventually bursts, then infect other phagocytic cells and continue the cycle. In cases of visceral leishmaniasis, all organs, containing macrophages and phagocytes, can be infected, especially the lymph nodes, spleen, liver, and bone marrow.

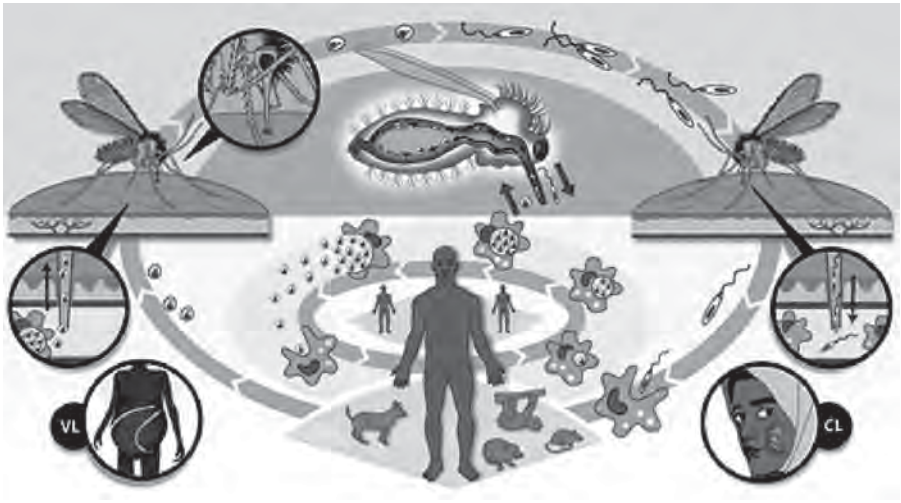

Fig. 6.4. *Leishmania* life cycle (WHO website: http://www.who.int/tdr/diseases/leish/leish.htm).

6.1.4 Symptoms

A high rate of infected people remain asymptomatic, but for others, the infection by *Leishmania* can produce very different clinical symptoms. Indeed, several forms of leishmaniasis exist: cutaneous leishmaniasis and mucocutaneous leishmaniasis, which cause skin sores, and visceral leishmaniasis, which affects some of the internal organs of the body (e.g., spleen, liver, bone marrow). People with cutaneous leishmaniasis usually develop skin sores a few weeks (sometimes as long as months) after being bitten, whereas people with visceral leishmaniasis usually become sick within several weeks or months (rarely as long as years).

The most severe form of the disease is visceral leishmaniasis (VL) (Fig. 6.7), which has a mortality rate of almost 100% if untreated. It is characterized by irregular bouts of fever, substantial weight loss, swelling of the spleen and liver, and anemia. *Leishmania* species responsible for this form mainly belong to the *Leishmania donovani* complex. VL caused by *L. infantum* especially affects children. Other symptoms, called post-kala-azar dermal leishmaniasis (PKDL), can appear several months (or years) after VL treatment. This complication of VL is characterized by a macular, maculopapular, and nodular rash in a patient who has recovered from VL and who is otherwise well [400].

Mucocutaneous leishmaniasis (MCL) (Fig. 6.8.), mainly caused by *L. braziliensis* and more rarely by the *L. guyanensis* complex, produces lesions that can lead to extensive and disfiguring destruction of mucous tissues of the nose, mouth, and body, including the face, arms, and legs, causing serious disability.

The cutaneous leishmaniases (CL) (Fig. 6.9) are the most common and represent 50–75% of all new cases. CL also result in a variety of clinical manifestations, in terms of the number of lesions (up to 200 on the exposed part of the body) and with selfhealing lesions compared with lesions requiring specific anti-*Leishmania* treatment. The lesion is localized at the site of the sand fly bite and satellite lesions in the vicinity of the original lesion can sometimes be observed. CL are mainly attributable to *L. amazonensis*, *L. braziliensis*, *L.*

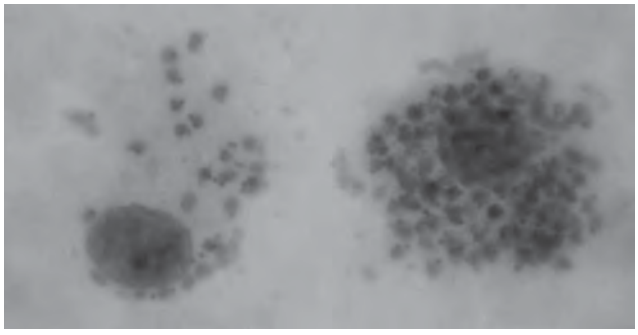

Fig. 6.5. Two human macrophages infected by *L. donovani* amastigotes, all rights reserved.

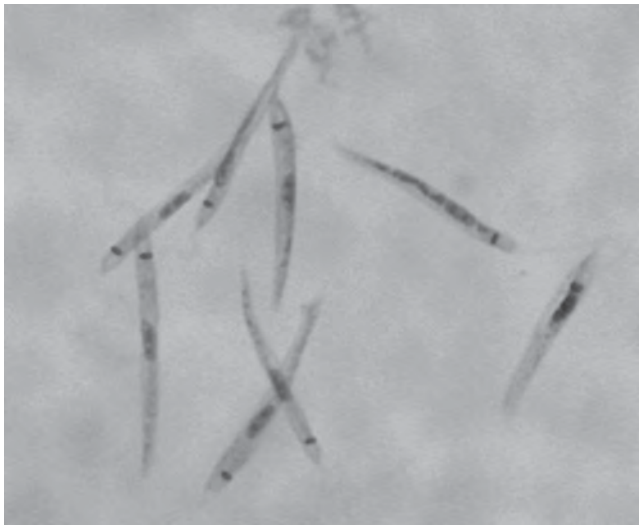

Fig. 6.6. *L. infantum* promastigotes, all rights reserved.

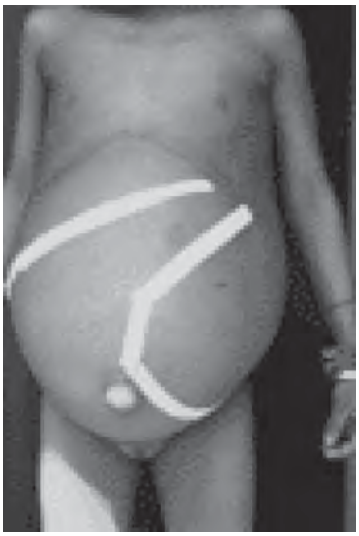

Fig. 6.7. Visceral leishmaniasis. (Photo taken by Philippe Desjeux. WHO website: http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html.)

guyanensis, *L. mexicana* (Biagi, 1953), *L. panamensis*, *L. naiffi*, *L. venezuelensis*, *L. lainsoni*, and *L. shawi* in the New World and *L. major* (Yakimoff and Schockor, 1914), *L. aethiopica* (Ashford and Bray, 1973), *L. tropica* (Wright, 1903), *L. arabica*, and *L. gerbilli* (Wang, Qu, and Guan, 1964) in the Old World, even if other species such as *L. donovani* (Laveran and Mesnil, 1903), *L. infantum* (Nicolle, 1908) have also been isolated from cutaneous lesions. Diffuse CL, mainly caused by *L. amazonensis* and *L. aethiopica*, never heals spontaneously and tends to relapse after treatment. This form is characterized by disseminated nodular lesions that resemble lepromatous leprosy.

Finally, these diseases have not only been found in developing countries since 1985, when the first co-infected patient was detected [93], even if the *Leishmania*–HIV co-infection cases are decreasing in Europe (introduction of Highly Active Antiretroviral Therapy (HAART)). These cases are mostly localized in Europe where intravenous drug users have been identified as the main population at risk. In this case, the immunological status of these people creates a favorable ground for the *Leishmania* parasite.

Fig. 6.8. Mucocutaneous leishmaniasis. (Photo taken by Philippe Desjeux. WHO website: http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html.)

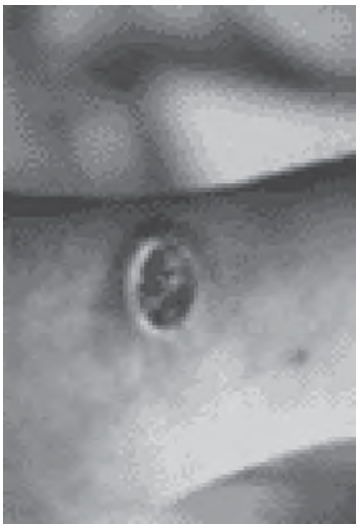

Fig. 6.9. Cutaneous leishmaniasis. (Photo taken by Philippe Desjeux. WHO website: http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html.)

6.1.5 Prevention, Diagnosis, and Treatments

Leishmaniases are a diverse and complex group of disorders. Unfortunately, strict rules cannot be applied for a type of *Leishmania* causing a typical disease, as even subtle changes in host immunity, the environment, and the parasite itself might result in completely different clinical manifestations; therefore, various approaches to disease control are necessary. Hence, prevention, diagnosis, and treatments depend on *Leishmania* species diagnostics and on the disease form; they differ for CL, VL, and MCL.

6.1.5.1 Prevention of leishmaniases

6.1.5.1.1 Zoonotic cutaneous leishmaniasis (ZCL) In the Old World, identification and control of animal reservoirs (small rodents) consist of deep plowing to destroy the burrows (breeding and resting sites) and plant (Chenopodiacae) sources of food for the rodents. Poisoning is no longer used, as it is considered too dangerous for other animals. In New World, especially in Latin America, large mammals living in forests or around houses can help contain the disease. In recent years, there has been an increase in the incidence of ZCL attributable to urbanization and deforestation, leading to domestication of transmission cycles, and the building of dams and new irrigation schemes, which have increased the population of animal reservoirs. Because populations living close to or at the edge of forests are particularly vulnerable, such habitats should be moved away from the forests. Limited clearance of peridomestic forest can reduce the risk of intradomiciliary transmission [101,102].

6.1.5.1.2 Anthroponotic cutaneous leishmaniasis Anthroponotic cutaneous leishmaniasis (ACL) is confined to urban or suburban areas of the Old World. Early diagnosis and treatment of recurring cases are necessary to avoid an increase in transmission risk, as they reduce morbidity, mortality, and transmission (reduction of human reservoir). The best prevention for ACL is the use of long-lasting impregnated bed nets in order to prevent infected sand flies from infecting healthy people and reduce untreated cases that continue infecting sand flies. Residual insecticide house spraying is another important prevention and intervention strategy. Mosquito repellents can be combined with pyrethroid-impregnated clothes (e.g., uniforms for military personnel) for individual protection [78,99]. Mosquito coils and the electrically heated fumigation mats containing pyrethroids are also helpful in protection.

6.1.5.1.3 Zoonotic visceral leishmaniasis In zoonotic visceral leishmaniasis (ZVL) endemic areas, the dog is a major reservoir. Several preventive measures are advocated: insecticide-impregnated dog collars, vaccination of pets against leishmaniasis, and elimination of infected stray dogs can decrease the incidence of infection. Canine and indirectly human leishmaniasis (because dogs are the *Leishmania* reservoir) is prevented by using deltamethrin-treated collars to protect dogs against *L. infantum* infection [127].

6.1.5.1.4 Anthroponotic visceral leishmaniasis (AVL) Elimination of the human reservoir by early diagnosis and treatment of PKDL and VL can reduce the transmission effectively. Furthermore, in anthroponotic foci, vector control through residual insecticide spray and improvement of the environment to control the growth of sand flies are the major tools for prevention.

6.1.5.2 Diagnosis of leishmaniases

6.1.5.2.1 Visceral leishmaniasis Typical clinical features of VL such as fever followed by splenomegaly (*enlargement of the spleen*) and lymphadenopathy (*swelling of the lymph nodes*) in a patient living in the endemic area should arouse suspicion of VL. Presence of antileishmanial antibodies, detected through conventional ELISA, IFAT, or DAT or the popular rapid rK39 strip test, indicates infection [2,12,20,117,149,331,354,385]. This is usually confirmed through demonstration of amastigotes in tissue smears mostly from the spleen, bone marrow, or lymph nodes. Polymerase chain reaction (PCR) is employed for demonstration of parasitic DNA in peripheral blood for diagnosis [249,309].

In India, a rapid strip test based on rK39 antigen has become available and should improve the diagnostic situation [42,136,250,353,354,375,401]. However, there is a need to develop a diagnostic test that has a high degree of specificity for active disease. Detection of antigen in urine (KAtex) is a promising tool, provided its format is improved [17,116,150, 279,312,345]. DNA detection by PCR is another powerful tool that could be established at several nodal centers in endemic areas serving the entire endemic region for diagnosis and evaluation of cure [214,248,249, 265, 308,327,394]. Both KAtex and PCR correlate well with disease activity and thus have a clear edge over tools based on antibody detection.

6.1.5.2.2. Cutaneous leishmaniasis In areas of endemicity without sufficient laboratory infrastructure, CL is often diagnosed on the basis of clinical characteristics of the lesions. However, parasitological confirmation is important, because clinical manifestations may be mimicked by other infections and granulomatous diseases: lupus vulgaris, leprosy, and so on. *Species identification may be important in predicting the course of the disease and selecting therapy*.

Leishmania may be isolated in up to 80% of sores during the first half of their natural course [273]. Parasites seem to be particularly difficult to isolate from sores caused by *L. braziliensis*, responsible for the vast majority of cases in Brazil. Touch preparations from biopsies and histopathology usually have a low sensitivity [81,389]. Slit-skin smears taken from the nodular edge of the lesion, or scrapings from within the ulcer [273] examined microscopically are positive in 32.7–84% [242,389]. Culture of fine needle aspiration material has been reported to be the most sensitive method [242,389]. Mucocutaneous leishmaniasis (MCL) is more difficult to diagnose parasitologically; even hamster inoculation only brings the yield up to 50% [389].

PCR introduced to determine the parasite species is used increasingly for diagnosis, greatly improving the diagnostic rates for CL and MCL [92,203]. For CL in Ecuador, using culture as standard, PCR was 97% sensitive as compared with microscopy (42%) and histology (33%) [18],whereas in Brazil, 71% of MCL cases were detected by PCR compared to 17% detected by conventional method [203]. Clinically, species identification may be important for epidemiological and therapeutic reasons, for example to identify the dominant species in a CL focus in Brazil [91]. Isoenzyme methods [283] and monoclonal antibodies [15,158] have been employed for species typing as well as analysis of amplified minicircle kinetoplast DNA (kDNA), by choosing primers from variable regions of different *Leishmania* species kDNA minicircle [327].

6.1.5.3 Leishmaniasis treatments

Treatment of leishmaniases has centered around *pentavalent antimonials* ($Sb^{V)}$ for six decades except in North Bihar, India, where large-scale antimony resistance is emerging and where Sb^{V}, even with the higher doses, is able to cure only 35–50% of patients [342,352,356,359,360]. In the Old World (*L. major*, *L. tropica*, and *L. donovani* complex) and the New World (*L. mexicana* and *L. braziliensis* complexes), CL and PKDL are commonly treated with Sb^{V}. A species-based approach to treatment has been advocated, especially in countries where several species may cause CL [245,295]. Intralesional Sb^{V} has been used with encouraging results in the Old World selfhealing CL [5,371].

A second-line drug, *pentamidine isethionate*, is expensive and toxic, beacuse it can be responsible for irreversible insulin-dependent diabetes mellitus and death. It was used to treat Sb^{V}-refractory patients with VL, but its efficacy has declined

and its use for VL has been abandoned [160,162,164]. During the late 1980s and the early 1990s, many Indian patients died for want of treatment after failing therapy with Sb^V and pentamidine. Though for some forms of CL, pentamidine is still attractive because very few doses are needed [9,335].

Due to increasing Sb^V-unresponsive VL, especially in India over the last decade, *amphotericin B* has become the drug of choice [230]. However, it is toxic and requires close monitoring. Though the cure rate with amphotericin B is approximately 100% and relapses are rare, the need for hospitalization lasting 5–6 weeks, infusion reactions, occasional serious adverse reactions such as hypokalemia, myocarditis, and death precludes its widespread application in peripheral health posts where monitoring facilities are limited. Thus, a large number of patients have to wait several weeks to months for hospitalization and treatment [132,229,230,361]. In South America, many regard amphotericin as the drug of choice for MCL, because of the low relapse rate [80,291]. The introduction of lipid-associated amphotericin, *i.e., liposomal amphotericin B (AmBisome), amphotericin B lipid complex (ABLC; Abelcet) and amphotericin B colloidal dispersion (Amphocil)*, has been one of the most important developments in the chemotherapy of leishmaniasis. In these formulations, deoxycholate has been replaced by other lipids that mask amphotericin B from susceptible tissues, thus reducing toxicity, and are preferentially taken up by reticuloendothelial cells, thus targeting drug delivery to the parasite and increasing efficacy. Three lipid formulations are commercially available, but their cost is prohibitive [88,89,106,188,227,351]. In India, all three formulations, with comparable efficacy, have been used, with AmBisome being the safest [89,105,188,343,344,346,348,349,351].

Paromomycin, an aminoglycoside, is well tolerated and effective for VL, but less so for CL [74,76,163]. Topical paromomycin ointment has been used for the treatment of CL [187,252,305]. The search for an effective oral antileishmanial drug spans two decades. *Allopurinol*, the azoles, rifampicin, and atovaquone showed activity in experimental systems, but proved disappointing in clinical trials. Oral *miltefosine*, an alkyllysophospholipid, originally developed as an anti-cancer agent, is now approved for the treatment of VL in India [347]. In several clinical trials, miltefosine cured more than 90% of patients with only minor gastrointestinal side effects such as vomiting in about half of the patients and less commonly diarrhea [165,347,350,355]. An asymptomatic transient rise in hepatic transaminases occurs during the second week of treatment, returning back to baseline values on continued treatment. It induces rapid cure, with a majority of patients becoming afebrile within the first week, quick regression of spleen, and recovery of blood counts. However, due to the risk of teratogenicity, Miltefosine should not be given to child-bearing age women except if contraception can be secured during and after treatment. Oral *sitamaquine*, an 8-aminoquinoline derivative, has been shown to have clinically significant antileishmanial activity. This effective oral antileishmanial compound has been tested in Kenya, Brazil, and India [104,161,325,387].

6.1.5.4 Vaccines? *There is no vaccine available* against any form of leishmaniasis for prophylaxis. Control of leishmaniasis remains a source of grave concern worldwide. As most of the available methods for leishmaniasis treatment and control are of limited effectiveness, there is now an urgent need for new low-cost drugs and/or new therapeutic interventions such as a vaccine, which would be the most practical and efficient tool for the control of these parasitic diseases [90].

Although considerable progress has been made over the last decade in understanding the immune mechanisms underlying protective responses, identifying potential candidate antigens, and implementing these principles in animal models, very few candidate vaccines have progressed beyond the experimental stage.

In recent years, great interest has been focused on the development of vaccines against localized cutaneous disease. Comparatively, VL has received limited attention. Indeed, only studies to identify the immunological factors of VL patients after chemotherapy and in asymptomatic subjects have been reported so far [231]. In regions where VL is endemic, such as the Mediterranean area, severe disease only occurs in a small population of around 10–33%, whereas the majority of infected individuals show no clinical symptoms and a significant part have self-resolving infection [21]. Furthermore, patients who have recovered from kala-azar are usually immune to reinfection, suggesting that *vaccination against VL should be possible*. The fact that a large proportion of the people living in endemic areas has self-resolving subclinical infections and the immunological mechanisms that control parasite multiplication in asymptomatic subjects are not well defined provides a rationale for designing immunoprophylactic strategies against VL.

Historically, "leishmanization" with live organisms was used to protect against disfiguring CL, because of the knowledge that individuals whose skin lesions had healed were immune. Knowledge of pathogenesis fortified by immunological understanding and genetic sequencing studies have gradually led to rational approaches toward the induction of protective immunity to *Leishmania* in animal models. Thus far, attempts at human vaccination have been unsuccessful, but several promising candidate vaccines are being explored in mouse models and in dogs.

In humans, measurement of cytokines in culture supernatants of *Leishmania* antigen-activated PBMCs and T-cell clone analysis support the view that (i) cell-mediated immunity, regulated by Th1 $CD4^+$ lymphocytes, was required for the destruction of *Leishmania* parasites in macrophage phagolysosomes [179]; (ii) control of infection in asymptomatic subjects was partially associated with the expansion of parasite-specific $CD8^+$ lymphocytes [211]; and (iii) these measurements revealed a coexistence of Th1 and Th2 responses in kala-azar patients as well as in cured individuals [253]. Therefore, even in humans, it is difficult to demarcate the responses leading to either visceral disease ("susceptible") or protective immunity ("resistance") against *Leishmania* parasites. Successful resistance

is probably the result of cooperation between the various arms of the immune system.

Recently, a vaccine against canine VL involving *Leishmania* excreted–secreted antigen has been developed (*Li*ESAp) [226]. It proved efficient in both experimentally and naturally *L. infantum*-exposed dogs in southern France [147,194]. In dogs, the vaccine-induced protection correlates with an early production of IFN-γ by a Th1 subset of $CD4^+$ T cells, which activate macrophages to destroy intracellular amastigotes through NO production. This was demonstrated by anti-*Li*ESAp IgG2 reactivity, *Li*ESAp-specific lymphocyte proliferation assays, and enhanced NO-mediated anti-leishmanial activity of canine monocyte-derived macrophages (CM-DM). In vaccinated dogs, NO-mediated *Leishmania* killing was associated with higher IFN-γ production by T cells when *L. infantum*-infected CM-DMs were co-cultured with autologous lymphocytes [147,194]. The main scientific issues in the design of a *Leishmania* vaccine are no different from those for any other vaccine. On a positive note, there is currently rapid progress in our understanding of the molecular nature of potential vaccine candidates and the mechanisms that determine infection-preventing immune responses. Multidisciplinary approaches integrating studies on parasite and host factors would facilitate our understanding of the disease and help in the design of a vaccine against human VL.

6.1.6 Why an Integrated Approach?

Even if we can generalize the life cycle of *Leishmania* because it always contains one vector, one parasite, and one host, the outcome of transmission, infection, and disease are dependent on the intrinsic characteristics of these three players. Indeed, *the epidemiology of leishmaniasis will be reflective of the particular combination of interactions among all players*: *parasite, vector, reservoir host, and environmental conditions*. In many endemic areas, the exact role of these players and their relations to human infections are unknown and it is difficult to generalize. Integrated analysis of both parasite genetics, parasite virulence factors, host immune responses, vector competence, host genetics, socioeconomic, and environmental risk factors is necessary for a better understanding of the interplay between these different factors and the risk of developing leishmaniasis. This approach could also provide information on the critical biological pathways involved in the host resistance or susceptibility to leishmaniasis and therefore help in orienting new therapeutic or vaccine strategies. Indeed, factors determining the host resistant/susceptible status are complex and largely unknown. Environmental factors acting on the phlebotomine and/or animal reservoir populations could modulate exposure of the human host to infected sand fly bites. Moreover, it has been suggested that the host immune response may also depend on the parasite strain, and different parasitic factors directly or indirectly responsible for the disease outcome have been described. Factors affecting the patient immune competence such as HIV infection or malnutrition have also been described to mediate susceptibility to VL. Immunity in leishmaniasis is considered mainly T-cell mediated, but more and more nonspecific factors acting in the early stage of infection are now considered as important for either the progression or control of the disease. Therefore, we will first expose the advances in the identification of the factors involved, due to the vector (Section 6.2), parasite (Section 6.3), and host (Section 6.4), and in the interactions between these players. The last section will focus on kala-azar in India, and we will demonstrate the necessity of this integrated approach to better understand this complex epidemiologic focus.

6.2 IMPACT OF SAND FLY VECTORS ON LEISHMANIASIS

Phlebotomine sand flies belong to the order Diptera, suborder Nymatocera, and family Psychodidae. They are small, about 3 mm in length, hairy flies characterized by a "hopping" flight and wings that remain erect above the abdomen when at rest. Sand flies are widely distributed and occupy tropical, subtropical, and temperate biotopes [4].

Phlebotomine sand flies are biological vectors of *Leishmania* in which the parasites undergo a complex developmental cycle beginning with ingested amastigotes and terminating with transmission of infective metacyclic promastigotes. Not all sand fly species transmit *Leishmania* parasites, however, with the genera *Phlebotomus* (Old World) and *Lutzomyia* (New World) accounting for all incriminated vectors to date. The bite of an infective sand fly vector is the only means by which any *Leishmania* species can be transmitted at a sustained and significant level. Importantly, the impact of sand flies on the establishment and spread of leishmaniasis extends beyond the transmission of *Leishmania* parasites to a direct effect on the host response to infection. In this section, the complexity of sand fly—*Leishmania* and sand fly—mammalian host interactions is outlined.

6.2.1 The Life Cycle of *Leishmania* in a Competent Sand Fly Vector

The life cycle of *Leishmania* parasites is contained within the digestive tract of the sand fly and begins with the ingestion of an infected blood meal containing amastigotes. Around 4 h after blood feeding, a chitinous *peritrophic matrix* (*PM*) is secreted, surrounding the blood meal within 24 h. The PM acts as a barrier that slows the diffusion of digestive enzymes secreted by the sand fly in response to blood ingestion and indirectly protects the parasites from the harmful effects of the enzymes [260]. This provides the opportunity for amastigotes to differentiate into sluggishly dividing procyclics, and by day 2 into large flagellated nectomonads (Fig.6.10). The blood meal is digested around 3–4 days after feeding. At this point, the PM breaks down, permitting escape of nectomonads and their attachment to the midgut epithelium. The degradation of the PM was initially attributed in full to the secretion of chitinases by *Leishmania* parasites [315]. Recently, however, Ramalho-Ortigao

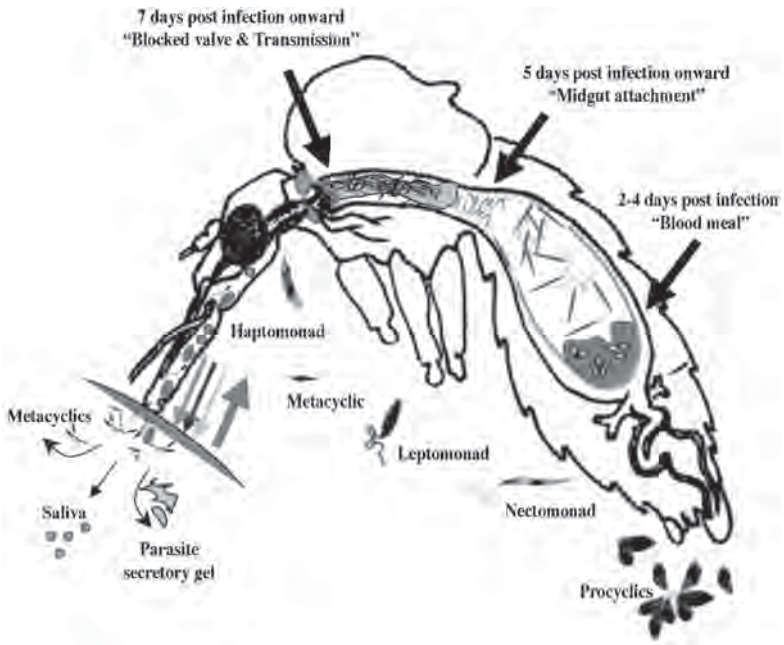

Fig. 6.10. Life cycle of *Leishmania* in a competent sand fly vector. See color plates.

et al. [272] showed that sand flies secrete their own chitinases after induction by the bloodmeal. The activity of sand fly chitinases peaks at about 48 h post blood feeding, coinciding with the time of the escape of nectomonads from the confinement of the PM [269]. Once in the gut lumen, attachment to the epithelium allows the nectomonads to persist in the midgut and prevents their expulsion with remnants of the undigested blood meal. Thereafter, nectomonads differentiate into leptomonads that divide rapidly as they migrate anteriorly to the thoracic part of the midgut [135,293]. Around day 7 after feeding, leptomonads give rise to infective metacyclics that accumulate in the anterior midgut below the stomodeal valve [135,293]. Metacyclics are characterized morphologically by their small cell body and long flagellum, and functionally by their free and rapid motility [311]. Simultaneously, haptomonads, highly specialized forms that adhere to each other and to the stomodeal valve, form a concentric parasite plug that blocks the opening of the valve (Fig. 6.10).

With such a complex life cycle, the parasites have to overcome several adverse conditions before they can successfully complete their development in the fly [180,299]. Such obstacles include digestive enzymes secreted by the sand fly [50,108,270], midgut lectins [381,382, 384], excretion of bloodmeal remnants [182,261], and sand fly innate immune responses [51,271]. As a result, different species of *Leishmania* closely evolved to fit distinct sand fly species, overcoming these obstacles and giving rise to the term "vector competence."

6.2.2 Vector Competence

A major determinant of vector competence is the ability of parasites to attach to the midgut epithelium of the sand fly to avoid expulsion with the blood meal remnants. Numerous studies, some involving mutants specifically deficient in *lipophosphoglycan* (*LPG*), a large and abundant molecule on the surface of *Leishmania* promastigotes, have implicated LPG as the ligand that mediates this attachment [62,262,302,303]. LPG is a tripartite GPI-anchored molecule with a backbone of conserved disaccharide repeats consisting of phosphorylated galactose-mannose sugars $-6Gal\beta 1,4Man\alpha 1\text{-}PO_4-$ capped with a neutral sugar. The LPG of different *Leishmania* species is highly polymorphic where the backbone can be unsubstituted (*L. donovani*, Sudan; and *L. chagasi*), partially substituted (*L. donovani*, India), or completely substituted (*L. major* and *L. tropica*) by side chains varying in the number and nature of their sugar residues [206,216,217,332,368] (Fig. 6.11A). The driving force for the observed LPG side chain substitutions is thought to be dependent on the complexity of the receptor present on the midgut epithelium of the targeted sand fly vector. Experimental infections showed that some sand fly species, such as *Lutzomyia longipalpis* and *Phlebotomus argentipes*, developed mature transmissible infections when infected with several foreign *Leishmania* species [168,261,294,304]. These species were termed *permissive vectors*. Others, including *P. papatasi* and *P. sergenti*, can only support the growth of the *Leishmania* species they are found infected with in nature (*L. major* and *L. tropica*, respectively) [168,261]; as such, they are considered *restricted vectors*. It is important to note that this species-restricted vectorial competence can also be strain specific. Certain natural variants of *L. major*, such as the West African Seidman strain, which lacks galactose side chains, do not maintain infection in *P. papatasi* but do maintain infection in another, closely related species *P. duboscqi* [206]. The strain-specific variability of LPG galactosylation in *L. major* was

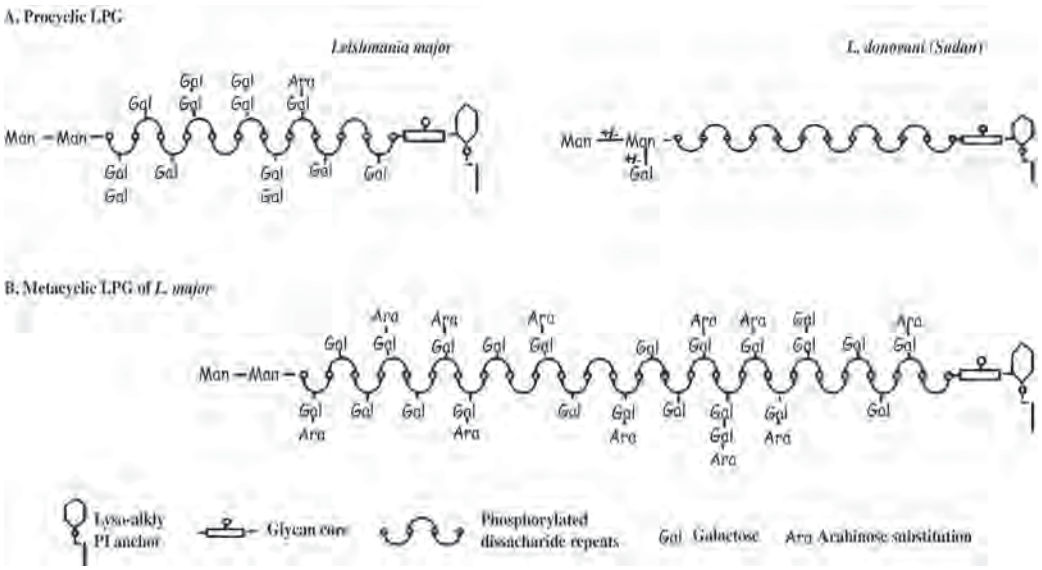

Fig. 6.11. Illustration of (A) *Leishmania major* (completely substituted) and *L. donovani* "Sudan" (unsubstituted) LPGs and (B) the changes during metacyclogenesis of *L. major* LPG.

attributed to the differential expression of a family of six genes encoding *L. major galactosyltransferases* that vary in their expression and activity [110]. Additionally, in the north of Israel, a strain of *L. tropica* whose LPG terminates with galactose instead of glucose residues, known to decorate the LPG of previously characterized *L. tropica*, was isolated from *P. arabicus* and not the classical vector *P. sergenti* [332]. As for sand fly midgut receptors, the first and only identified receptor to date is *PpGalec*, a tandem repeat galectin responsible for the observed specificity of *P. papatasi* for *L. major* [169].

Though appropriate LPG polymorphisms are necessary, vector competence has also been associated with the ability of certain *Leishmania* species to overcome other adverse conditions in the midgut of their respective competent vectors. For example, *Leishmania* species are able to overcome the harmful effects of digestive enzymes in a competent vector, but not in a foreign sand fly species, by specifically inhibiting or retarding the peak activity of these enzymes [50,107,316]. Secreted glycoconjugates, a family of LPG-related molecules characteristic of *Leishmania*, were implicated in this protection [302,317], highlighting the degree of adaptation necessary for parasite survival in competent vectors.

6.2.3 Metacyclogenesis and Transmission

Transmission of the parasites from the sand fly to the mammalian host requires detachment of the parasites from the midgut epithelium. This event is again mediated by *LPG*, which undergoes *stage-specific modifications* involving elongation of the molecule and/or changes to the nature of sugar residues on its side chains or neutral cap [206,217,262,301,303]. For example, during metacyclogenesis, the LPG of *L. major* elongates to approximately twice its procyclic length, and the majority of terminal galactose sugars get capped by arabinose residues (Fig. 6.11B). This modified LPG cannot bind to PpGalec, the midgut receptor for *L. major* procyclic LPG in *P. papatasi* [169]. In *L. chagasi*, metacyclics downregulate the glucose substitutions in their LPG, which, in contrast to procyclic parasites and procyclic LPG, becomes unable to bind to the midgut of its natural vector *L. longipalpis* [333]. This detachment frees the metacyclics and ensures their availability for transmission to the mammalian host. The trigger that initiates metacyclogenesis is not well understood. The only available evidence to date is a negative regulation by tetrahydrobiopterin, a byproduct of pteridine metabolism, whose levels are high following a bloodmeal and decline with time elevating metacyclogenesis [84].

To further enhance their chances for successful transmission, *Leishmania* parasites evolved the haptomonad stage, whose specific function is to block the stomodeal valve separating the midgut from the foregut. These parasites are nonmotile and adhere to the chitinous lining of the valve. The physical blockage of the valve is compounded by the secretion by the parasites of a proteophosphoglycan-rich gel termed the *promastigote secretory gel* (*PSG*) [156,340]. Both act in concert to obstruct the intake of blood during feeding, requiring more bites and a longer period to feed, and promoting *regurgitation of metacyclics* into the skin of the mammalian host [33,183,294]. In addition, parasite chitinases destroy the chitinous lining of the stomodeal valve, further contributing to the defective feeding mechanism in infected flies [314,380]. Another aspect of sand fly feeding that promotes transmission is sand fly probing. Due to their small mouth parts, sand flies need to lacerate multiple skin-surface capillaries to create the pool of blood upon which they feed [277,278]. Beach et al. [32] have shown that infected sand flies can transmit *Leishmania* parasites while probing. Moreover, infected flies

with mature infections and a stomodeal valve destroyed by chitinases and blocked by haptomonads and PSG probe longer in their efforts to feed [33,293,314], thus further promoting transmission. Based on a pool of 50 flies, the number of metacyclics egested into a membrane feeder by an infected sand fly was averaged at 1000 parasites [294]. However, considering the modification of feeding behavior mentioned above, the full potential of transmission in infected sand flies has yet to be accurately defined.

6.2.4 Sand Fly Modulation of the Mammalian Host Immune Response

6.2.4.1 Sand fly saliva During the act of probing and feeding, sand flies salivate into the wound. Consequently, *Leishmania* metacyclics are always egested in the presence of saliva. Sand fly saliva consists of a complex mixture of pharmacologically active compounds such as vasodilators, anticoagulants, and platelet inhibitors [166,278,372], as well as a number of immunogenic proteins of unknown function [72,246,373]. Numerous studies have shown that *sand fly saliva enhances* Leishmania *infections and has the ability to modulate the host immune response* (reviewed in [131,166,299]). Moreover, preexposure to saliva protected mice against infection with *L. major* [37,167]. Therefore, salivary molecules identified as disease enhancing or immunogenic may be targets for vaccine development. *Maxadilan*, a vasodilatory peptide identified from *Lu. longipalpis* [195], and *SP15*, a salivary molecule of unknown function identified from *P. papatasi* [373], both protected mice against infection with *L. major* [235,373]. Currently, salivary proteins of various sand fly vector species are being evaluated for their ability to protect against the *Leishmania* species they transmit in nature. This is made possible by the development of a high-throughput approach to DNA plasmid production combined with an immunization strategy that accelerates the identification of salivary molecules producing a cellular response, an antibody response, or a combination of both [246].

6.2.4.2 Promastigote secretory gel PSG is produced by leptomonad forms of *Leishmania* and accumulates at the anterior midgut region of an infected sand fly where it is egested with metacyclics during transmission by bite [31,293]. *Filamentous proteophosphoglycan* (*fPPG*), a component of PSG, was found to enhance *L. mexicana* infection in mice, causing long-term disease exacerbation [294]. Again, an intimate adaptation of *Leishmania* parasites to their vectors is reinforced, where molecules of parasitic origin and delivered by the fly insure the successful transmission of *Leishmania* and its establishment in its mammalian host.

6.2.4.3 Conclusions The role played by phlebotomine sand fly vectors in the development of *Leishmania* parasites, their successful transmission, and the outcome of disease is substantial. The complexity of the life cycle of *Leishmania* parasites in the digestive tract of the sand fly, from surviving the onslaught of digestive enzymes and immune molecules to attaching to receptors on midgut epithelial cells, exerts a powerful evolutionary pressure that restricts the species of *Leishmania* that can be successfully transmitted by a particular species of sand flies. In some instances, as for *L. major* and *P. papatasi*, the specificity of this vector–parasite association is so reliable as to enable the identification of the vector following characterization of the *Leishmania* species circulating in a focus of disease. Appreciation of the full significance of sand flies as vectors of leishmaniasis came with the demonstration of their influence on the progress and outcome of disease in the mammalian host that extends beyond their delivery of parasites. Sand flies can alter the immune response of the mammalian host through the modulatory effect of molecules they inject into the skin. These include salivary molecules and/or molecules of parasite origin, such as PSG. Some of the most exciting fields of research today pertain to an integrated approach in the search for an effective anti-*Leishmania* vaccine that combines protective salivary molecules with *Leishmania* antigens. Taking all of the above into consideration, further research is needed to identify the key molecules involved in *Leishmania* – sand fly interactions, from those important to the survival of the parasite within the digestive tract of the vector to those influencing their transmission and establishment in the mammalian host.

6.3 BIODIVERSITY AND GENETICS OF PARASITES: IMPLICATIONS IN VIRULENCE AND PATHOGENICITY IN HUMANS

6.3.1 *Leishmania* Species and Epidemiological Diversity

The *Leishmania* (Ross, 1903) parasites are protozoa belonging to the Kinetoplastida order (Honigberg, 1963) and to the *Leishmania* genus. Kinetoplastida have a unique mitochondria-like organelle called the *kinetoplast*, an appendix of their single mitochondrion, located near the basal body of the flagellum. As described above, this genus is characterized by ecological, epidemiological, and clinical complexity. The presence of these organisms throughout the world, except Antarctica, and their capacity to infect a large range of vertebrate hosts and sand fly species shows that *Leishmania* spp. have the ability to adapt and survive in very diverse environments. The hypothesis based on epidemiological data is that almost all *Leishmania* hosts are adapted to these environments, and the infections remain inapparent [189]. On the contrary, within animals that are less well adapted, such as humans, infections can produce a wide range of diversified pathologies, from asymptomatic carriers and benign cutaneous lesions to more serious cases such as the visceral form (see Section 6.1.4. for details). Indeed, when humans are bitten by a sand fly, the parasite inoculation can lead to the development of leishmaniasis but can also have no incidence on humans. The rate of asymptomatic carriers (infected individuals without clinical manifestations) is not accurately known, but different studies have revealed that it

seems to be higher than expected. For example, on the Balearic Islands, *L. infantum* was amplified by PCR in 22% of blood donors [280] and asymptomatic carriers were also revealed in Brazil [77], southern France [192], and India [323].

This great phenotypic variability is also expressed by the high number of *Leishmania* species described in the literature. A large part of these species has been defined on the basis of epidemiological, clinical, geographical, and biological data, for example, *L. guyanensis* (isolated in Guyana), *L. peruviana* (isolated in Peru), *L. infantum* (isolated from a child in Tunisia), *L. gerbilli* (isolated from gerbils), and so on. These extrinsic characteristics were first used to determine the species because morphological characteristics cannot be used for species identification. Even if differences in length have been observed among *Leishmania* spp. [125,174], the different species are indistinguishable in morphology in both the promastigote and amastigote stages. The development of genetic and phenotypic tools has provided means to reconsider the *Leishmania* taxonomy more rigorously. The first problem noted was that *these organisms could not be defined on the basis of the biological concept of species* [215]. Indeed, the studies of population genetics published show a basic clonal population structure in different species [25,23,362,364,365]. However, this model is not as simple as it appears because these organisms have been shown to use different multiplication strategies, with several *hybridization events* between species evidenced in the literature [24,41,112,120,175]. For example, in the New World, hybrids between *L. braziliensis* and *L. peruviana*, and *L. guyanensis* and *L. braziliensis* were described [24,112], and in the Old World, hybrids have been shown between *L. major* and *L. arabica* [120]. However, these recombination events do not seem frequent enough to disturb the *clonal propagation* of clones stable in space and time. Thus, the species definition of these "agamospecies" (*a group of individuals in which reproduction is almost exclusively done by asexual means*) still remains arbitrary and is based on a mix of intrinsic and extrinsic characteristics considered together. In this framework, different analyses clearly showed that the species status of some taxa was not taxonomically valid or questionable [23,26,85,137,212,213,283,396].

It must be kept in mind that there is a need for a rigorous and clear nomenclature for efficient communication between the scientific and medical professions. Indeed, first the various *Leishmania* species require different medical posologies to treat patients (see Section 6.1.5.3 for details) and second, clinical data suggest *a close association between the clinical outcome of the disease in humans and the species responsible for the infection*. Concerning the second point, for examples, (i) the *L. donovani* complex is mainly responsible for visceral forms; (ii) mucosal lesions are generally associated with *L. braziliensis*; (iii) *L. major*, *L. tropica*, *L. mexicana*, *L. guyanensis*, and *L. peruviana* produce a variety of *Leishmania* skin lesions in humans; and (iv) *L. amazonensis* is generally associated with diffuse cutaneous leishmaniasis. But once again, the clinical picture is more complex since at an intraspecific level, we can observe different disease outcomes: for example, *L. amazonensis* was isolated from six patients, three with cutaneous lesions, one with mucosal lesions, and two with diffuse cutaneous forms [205]; *L. infantum* can cause both cutaneous and visceral forms; and *L. braziliensis* produces cutaneous lesions and in around 10% of cases metastasizes.

Other points complicate the clinical picture: the existence of hybrids (see above) and mixed infections with different *Leishmania* strains. Concerning hybrids, *L. braziliensis* can produce cutaneous or mucocutaneous lesions in humans requiring care, whereas *L. peruviana* is responsible for dry benign cutaneous lesions that heal spontaneously. The hybrids between these two species found in Peru were isolated from patients either with mucocutaneous lesions or with benign lesions typical of the *L. peruviana* species [112]. These strains are thus capable of producing the different pathologies found in each species. Concerning mixed infections by different *Leishmania* species, few cases have been described in the New and Old World in the literature [13,30,154,210,341]. However, the molecular epidemiology studies evidenced that many foci exist in which several species circulate simultaneously [205]. It is hypothesized that the number of mixed infections is underestimated because of a selection problem during the parasite culture required by molecular techniques. This is confirmed by a study conducted in Bolivia [30] and also presents a problem for *Leishmania* diagnosis, prognosis, and for the understanding of the real role of parasites in pathogenicity in humans.

Moreover, it seems important to note and to consider the cases of co-infection of *Leishmania* with other pathogens. This is relatively frequent according to the literature and various pathogens in association with *Leishmania* such as *Mycobacterium tuberculosis* [94,386], *Trypanosoma cruzi* [30], *Salmonella* and *Schistosoma* [109], and of course HIV (for reviews see [97,103,234,268]) have been studied. Furthermore, in some cases, these co-infections can produce unusual clinical forms of leishmaniasis [66,75].

Another aspect of the incredible environmental adaptation of *Leishmania* parasites is their ability to become drug resistant. Indeed, drug and multidrug resistance has emerged as a major problem in treating both VL and CL. In particular, the appearance of antimonial resistance has changed the pattern of leishmaniasis treatment in the world. Indeed, pentavalent antimony has long been the cornerstone of anti-*Leishmania* chemotherapy, but resistance to this drug class is so high in some parts of the world, particularly in northeast India (see Section 6.1.3 for details), that it is quickly becoming obsolete [251,352]. There are many factors that can influence the efficacy of drugs in the treatment of leishmaniasis. These include both an intrinsic variation in the sensitivity of *Leishmania* species, described for pentavalent antimonials, but also paromomycin, azoles, and other drugs that have reached clinical trials, as well as acquired drug resistance to antimonials [79]. Thus, *the understanding of the molecular mechanisms that the parasite adopts or may adopt in the future is of high clinical relevance*. We know that the parasite is able to adapt itself to become resistant. For example, some results on glibenclamide-resistant

Leishmania parasites suggest that drug resistance involves a metabolic adaptation that promotes a stage-dependent modulation of energy substrate uptake and use as a physiological response to the challenge imposed by drug pressure [370]. Resistance of *Leishmania* species, in many instances, is due to overexpressed efflux pumps belonging to the superfamily of ABC (ATP-binding cassette) transporters [193].

6.3.2 Different Pathogenic Potential of Species and Within Species: Experimental Data

From all these data, it seems clear that the clinical outcome of the disease in humans is multifactorial. However, despite the complex clinical picture, *the parasites play an important role in human pathology and are not a passive organism*.

The animal models are largely used for immunobiology studies to understand and characterize the host–parasite interactions during infections. The fact that different human parasite isolates produce different infection patterns in a given mouse model suggests that parasite-related factors play an important role in the resistant versus susceptibility status and in the type of immune response elicited by the infected host [173]. The studies showed that animal models such as mice, hamsters, or nonhuman primates respond differently depending on the *Leishmania* species used [220,392]. For example, the *Leishmania* (*Viannia*) subgenus (which are predominant in Latin America), fail to reliably infect mice [220]. Moreover, different experimental data also showed that at an intraspecific level (within species), different strains can have different levels of virulence or different pathogenic properties. Indeed, it was demonstrated in BALB/c IL-4-deficient mice that a particular *L. major* strain induced a non-healing infection, whereas a different *L. major* strain induced a healing infection [186,244] and thus different *L. major* strains can induce somewhat different host immunologic responses [151] in mice. Another example was based on the comparisons of infection in both mouse and hamster models using *L. tropica* metacyclics purified from dermotropic and visceral isolates [200]. They found differences in disease progression that may reflect the parasite tissue tropism and pathogenic potentialities displayed by these strains in their human hosts. The authors suggested a role for parasite-related determinants in the clinical spectrum of disease. Thus, it was shown that in addition to the host factors, parasites also influence susceptibility and immune response following infection.

6.3.3 Genetic Markers and Parasitic Factors Involved in Pathogenicity in Humans

Since the development of molecular tools, scientists have attempted (i) to determine whether there is a *Leishmania* phenotypic or genetic association with virulence of strains and/or with pathogenicity observed in humans and (ii) to identify the markers involved directly or indirectly in the clinical outcome of the disease.

Different direct and indirect parasitic factors influencing disease outcome have been described. These factors were classified into three types: (i) *indirect genetic markers of pathogenicity*, (ii) factors called *invasive/evasive determinants* by Chang and McGwire [71], and (iii) factors called *pathoantigenic determinants* [71]. It should be noted that the distinction between the different groups is somewhat unclear and must not be considered inflexible. Indeed, this classification depends on knowledge acquired on each type of marker and thus it could be questioned in the future.

6.3.3.1 Indirect genetic markers *Indirect genetic markers* regroup genes or loci not directly involved in virulence or pathogenicity; they have been and continue to be widely explored. Different molecular tools such as multilocus enzyme electrophoresis (MLEE), which is the gold standard method for species identification [283], random amplified polymorphic DNA (RAPD)[82,157]. Pulse field gel electrophoresis (PFGE) [113,139], restriction fragment length polymorphism (RFLP) on various gene [82,377], and recently microsatellites [60,159,298] and real-time PCR [267,320], were used and the data were compared with clinical and epidemiological data. This kind of comparison is justified because of the clonal model (see Section 6.3.1 for details) of these organisms [363]. Indeed, the frequency of genetic exchanges (absent or rare for clonal species and frequent or obligatory at each generation for sexual species) conditions the interest of these genes or locus as epidemiological or clinical markers. The clonality implied linkage disequilibrium (nonrandom reassortment of genotypes occurring at different loci) and thus, correlation between independent genetic and phenotypic markers, suggesting strongly the possibility to find some genotypes associated with clinical or biological phenotypes [226]. Genetic markers are numerous to distinguish the different species but only a few of them were found to be associated with various clinical phenotypes at the intraspecies level. For example, within *L. peruviana*, we found a link between MLEE data and severity of lesions in patients [23,111,114]; for *L. infantum*, some zymodemes (all the stocks pertaining to a zymodeme have the same MLEE patterns) were associated exclusively with dermotropic strains and others with strains mainly isolated from visceral forms of the disease [14,142,282]. Other investigations studying different genetic markers showed also a correlation between clinical polymorphism and genetic data in *L. infantum* [139] and in *L. braziliensis* [319]. But finally, these correlations remain weak and do not allow us to understand the role of parasites in the outcome of the disease and to use these tools as prognosis markers.

6.3.3.2 Invasive/evasive determinants Chang and McGwire [71] have identified a second group of markers called *invasive/evasive determinants*. They belong to parasitic mechanisms that are necessary to establish leishmaniasis such as (i) *Leishmania*–macrophage attachment; (ii) the entry of *Leishmania* into macrophages; (iii) intramacrophage survival; and (iv) differentiation and intracellular multiplication of *Leishmania* amastigotes, but these *invasive/evasive determinants are not responsible for the symptoms of the disease*. Thus, they refer

to all determinants that help successfully establish *Leishmania* infection in the host such as *glycosylphosphatidylinositol* (*GPI*), *glycosylphospholipid* (*GIPL*), *lipophosphoglycan* (*LPG*), *leishmanolysin* (*GP63*), *cysteine proteases* (*CPs*), among others. These molecules have been widely studied, especially LPG, GP63, and CPs. *LPG* is the dominant surface molecule of promastigotes involved in (i) binding, migration, and release of the parasite in the sand fly midgut but also in (ii) the modulation of resistance to lysis by the host's complement. It is almost completely absent from amastigotes [83,217,247,262]. LPG is not involved in virulence within all *Leishmania* species. For example, it is not required for infection by *L. mexicana* [155], whereas it is needed for *L. donovani* and *L. major* infection [221,337]. Its structure varies between *Leishmania* species and also differs between procyclic and metacyclic promastigotes (see Section 6.2.2 for details). Some analyses showed, in addition to stage-specific and interspecies variability, an intraspecies polymorphism in lipophosphoglycan structure [206]. This diversity may be linked to the *Leishmania* adaptation to the sand fly species rather than related to the clinical diversity observed in humans.

Another important surface molecule, *GP63*, is an ecto-metalloprotease particularly abundant in promastigotes and also released by this stage of *Leishmania* [219]. Like LPG, GP63 is downregulated in the amastigote form [318]. These molecules may be involved in the evasion from humoral lytic factors and in the attachment of parasites to macrophages followed by their intracellular entry into these phagocytes [395]. GP63 protein is encoded by a multigene family repeated in tandem. Genetic and structural diversity was extensively studied and showed a high polymorphism at both inter- and intraspecific levels [119,140,289,339,376]. Like LPG, this protein seems to be subjected to strong host-selection pressure by the vector as well as by the vertebrate host [141]. But no link was found between the genetic or phenotypic diversity of GP63 and the intraspecies clinical polymorphism of strains [139].

Scientists have also shown increased interest in *cysteine proteases* because of the key roles some of them play in infection and expression of the disease, making them potential drug targets or vaccinal antigen. In *L. major*, a total of 65 CPs may exist, many of which are likely to play crucial roles in host-parasite interactions, particularly in facilitating survival and growth of parasites in mammals by destruction of host proteins, nutrition, evasion of the host immune response, and *Leishmania* survival within host macrophages [4,237,240,297]. The functional studies of the most widely studied CPs, CPB, allowed to explore the ways in which these molecules influence the interactions between parasite and mammalian host (see reviews [4,237,240,297] for details). Indeed, the generation of *Leishmania cp*-deficient mutants and inoculation on mice showed the involvement of these proteins in virulence and pathogenicity. For example, the *L. mexicana* strain deficient in the *cpb* array reduced virulence in BALB/c mice [4,238]. As for the *gp63* array, the genetic studies showed a high level of polymorphism, among species as well as within species. Nevertheless, only one publication showed a statistical correlation between gene organization of *cpb* in the *L. infantum* population and the strain tropism (cutaneous versus visceral) [67].

This list is far from exhaustive: other molecules such as *PSA* (GP46), an abundant surface glycoprotein of the promastigote form [35,357], or *A2* protein, shown to have an influence on the outcome of the disease [398], appear to play an important role in the invasive/evasive phases of the *Leishmania* cycle. For example, A2 is an important gene for *L. donovani* virulence but is not expressed in *L. major* [397,398]. Nevertheless, we can note once again the high level of heterogeneity depending on the considered species.

6.3.3.3 Pathoantigenic determinants The third group of factors comprises *Leishmania pathoantigenic determinants* [71]. *This group includes all the molecules described in the literature capable of inducing host immunopathology as the principal cause of clinical symptoms*. Thus, all *Leishmania* antigens eliciting antibodies at high titers compared to antibody titers against the other determinants (invasive/evasive determinants) can be classified in this category. These pathoantigenic determinants are all conserved structural or soluble cytoplasmic proteins, which are often complexed with other molecules to form subcellular particles [71]. Moreover, they have been found to contain immunogenic B-cell epitopes. The list of candidate molecules is based on data obtained from kala-azar patients (the visceral form of the disease as described above) [276]; thus, they clearly differ from those obtained from cutaneous leishmaniasis. For example, the unique 117-basepair repeat, encoding for a 39-amino acid peptide (recombinant products = rK39) in the *Leishmania* kinesin-like gene, is expressed by the amastigotes of visceralizing *Leishmania* (*L. donovani*, *L. chagasi*) and not by dermotropic species (*L. major*, *L. amazonensis*, and *L. braziliensis*) [61]. Indeed, sera from kala-azar patients contains antibodies specific to this 39-amino acid peptide called anti-rK39 at high titers [331]. It is interesting to note that this antigen has been successfully used for serodiagnosis of active kala-azar cases.

To date, the interactions between these molecules and the human immune system as well as activation of specific antibodies production remain unknown. All these molecules are localized in amastigote cytoplasm and are thus beyond the reach of their specific antibodies [71]. However, their potential contributions to immunopathology are apparent. In a study on protective immunity in *Leishmania* [266], Chang and McGwire [71] suggest that some *Leishmania*-specific T-cell epitopes may also exist and cause additional immunopathology.

6.3.3.4 Conclusions In summary, all the experimental and epidemiological data show that the identity of the parasite responsible for infection plays a fundamental role in the clinical diversity observed in humans, as it does when we consider the different species as different parasites of a single species. As described above, *factors or factor groups from the* Leishmania *parasite could clearly be involved in this clinical diversity at both interspecific and intraspecific levels*.

Unfortunately, their true roles and the biological pathways in which they participate remain unknown because of the immunopathological complexity involved. All these studies are based mainly on the comparison of strains responsible for different degrees of pathogenicity. Nevertheless, it seems that the majority of infections remain inapparent in natural populations considering all the vertebrate hosts, but this is also true in humans, as described in the literature [77,209, 255,280]. Although it is known that leishmaniasis is the result of a complex association of host and parasite factors, we do not know what occurs in *asymptomatic carriers*. We do not know whether strains from patients and from asymptomatic carriers are genetically different. To explore the pathogenic potential of strains and identify the parasite factors involved in pathogenicity, it is fundamental to *compare isolates from asymptomatic carriers with parasites from patients*.

As described above, leishmaniasis results from apparently multiple factors of *Leishmania* origin but also host and environmental origin combined (see the other parts of this review for details). Thus, all these data illustrate the value of mechanistic approaches focusing on both parasite and host defense pathways in dissecting the specific biological roles of the different complex virulence factors and pathoantigenic determinants [338].

6.4 THE IMMUNE RESPONSE AND GENETIC FACTORS FROM THE MAMMALIAN HOST

In endemic area populations, it is striking to observe, for a given parasite species, a wide range of interindividual variability in susceptibility/resistance to disease. Furthermore, epidemiological studies have shown that infection by *Leishmania* parasites remains asymptomatic in most cases [21,57,146,399]. These subjects (detected either by a positive serology, the Leishmanin skin test, or detection of parasite by PCR [330]) are either able to clear infection or can remain asymptomatic carriers for years (as evidenced by the development of leishmaniasis in immunosuppressed patients several years after their last stay in endemic areas). Other subjects, however, are unable to control parasite dissemination and/or multiplication and develop clinical symptoms of diverse severity. Malnutrition, immunosuppression (AIDS, malignancy), pregnancy, age, as well as immunological capacities and genetic factors are risk factors associated with the development of leishmaniasis. Malnutrition alters the immune response and leads to increased parasite visceralization during *Leishmania donovani* infection [11,143]. Leishmaniasis in HIV-infected individuals is often the consequence of a reactivation of a latent infection. Accelerate multiplication of parasites and the invasion of multiple visceral sites stems from progressive T-cell immunosuppression [6,393]. *Leishmania*-HIV co-infections appear to be accompanied by changing nonpathogenic into pathogenic strains, and dermotropic strains are seen to induce viscerotropic behavior [7].

Although the general state of health and physiological conditions of the host can and do influence disease progression, genetic predisposition indubitably plays a major role in determining disease outcomes. Thus, the aim of this section is to analyze how the host response to parasite infection mediates susceptibility/resistance to leishmaniasis. First, the different host immunological responses to infection and their relation to susceptibility/resistance to disease will be presented, and then we will focus on how these observed response differences are related to genetic factors from the mammalian host.

6.4.1 The Host Immune Response to *Leishmania*

In their mammalian host, *Leishmania* species are obligate intracellular parasites of hematopoietic cells of the monocyte/macrophage lineage. As such they infect and multiply within cells having a central role in the host immune response, as they are both involved in *innate immunity* (as anti-*Leishmania* effector cells) and in presenting parasite antigens to lymphocytes, and thus in initiating the *acquired immune response* [95,96,300,334] (Fig. 6.12).

6.4.1.1 Early events On infection, *Leishmania* parasites are first confronted with the host's *innate immune response* (see Fig. 6.12). Mechanisms of the innate response leading to the control of infection are mediated by the intrinsic capacity of macrophages [133] to become infected by promastigotes and then by amastigotes and to activate on infection to limit parasite multiplication. The ability of macrophages and dendritic cells [40] to produce interleukin-12 (IL-12) and other pro-inflammatory cytokines (tumor necrosis factor-α [TNF-α], IL-1) early during the course of infection is also a critical step [358,366,367]. IL-12 has a key role in the development of cell-mediated immunity through the induction of naive T cells to differentiate into Th1 cells (see acquired immunity below) and through the activation of NK cells to secrete interferon-γ (IFN-γ) [36,313]. IFN-γ and TNF-α are cytokines involved in the activation of infected macrophages, which is characterized by an increased production of radical oxygen and nitric oxide (NO), which are potent anti-*Leishmania* molecules [49,121,122,148,196,241]. Intramacrophagic radical oxygen (ROS) is produced by the NADPH oxidase complex, whereas NO is produced by the inducible nitric oxide synthase (iNOS).

However, in no way can the parasite be seen as a passive partner in the establishment of the immune response. Indeed, several studies on macrophage gene expression have shown that the pattern of gene expression in infected macrophages is profoundly modified upon infection [54,73,292]: a number of genes encoding molecules involved in the macrophage anti-microbial response are down-modulated [53,68,100, 144,259,275,369,388], whereas fewer genes coding immunosuppressive molecules such as TGF-β, IL-10, IL-10R, are selectively up-regulated [29,48,59,123,138,383].

6.4.1.2 Acquired immunity *Acquired response develops with the surface parasite peptide-presentation by infected macrophages and dendritic cells* (see Fig. 6.12). These peptides are the result of

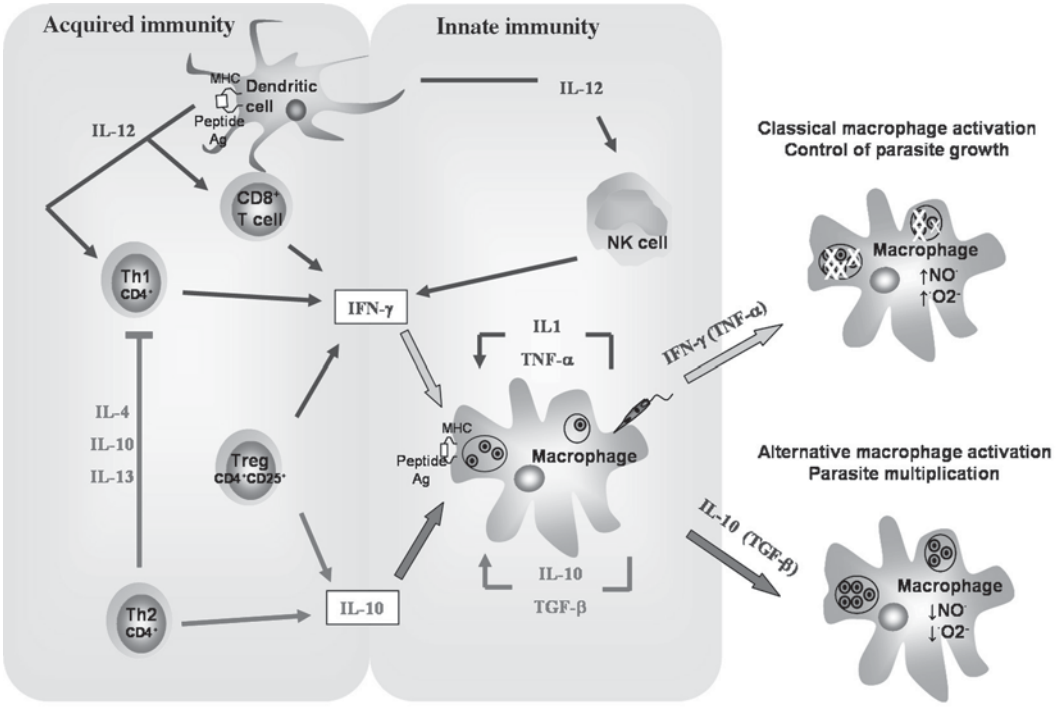

Fig. 6.12. Immunological determinants influencing parasite multiplication. During blood meal infected sand flies transmit metacyclic promastigotes to the vertebrate host, which convert to the amastigote form on entering macrophages and dendritic cells. IL-12 production from infected cells induces NK cells activation, CD4+ T helper cell differentiation, activation of CD8+ cytotoxic T-cells and INF-g production. INF-g stimulates iNOS expression and NO production in the macrophage, which mediates parasite killing. Failure to produce IL-12, to respond to INF-g or alternatively IL-4/IL-13 production results in unregulated parasite replication within the infected cells facilitated by host cell IL-10 production. IL-10 production by CD4+ CD25+ regulatory T-cells can both facilitate disease development as well as maintaining latent infection and concomitant immunity. See color plates.

intracellular processing of *Leishmania* antigens and are presented to T-cell receptors by the major histocompatibility complex (MHC) molecules [185,402]. Depending on the peptide presented and the cytokine context (i.e., presence of IL-12 or IL-4), this will lead or not to the activation and proliferation of $CD8^+$ cytotoxic T cells and to the differentiation of naive $CD4^+$ T helper (Th) cells into Th1 or Th2 subtypes [98,184,236]. *Th1* cells secrete cytokines usually associated with *inflammation* such as IL-2, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) and *induce* Leishmania *cell-mediated immune responses* (induction of macrophage microbicide activities and activation of cytotoxic T cells). In contrast, *Th2* cells help in the development of the *humoral* response (production of antibodies by B cells) and produce cytokines (IL-4, IL-5, IL-10, IL-13, etc.) that *inhibit* both *development of Th1 responses and macrophage activation* [274].

Other T-cell populations were shown to be involved in long-term protection. IFN-γ producing $CD8^+$ T cells or $CD8^+$ T cytotoxic cells play a role in immunity to reinfection [39,86,239]. More recently, $CD4^+$ $CD25^+$ regulatory T cells [306, 326] (*Treg* cells) were proved to mediate persistence of *L. major* parasites at a low level in healed cutaneous lesions [38]. Thus, Treg cells seem to suppress the ability of the immune response to completely eliminate parasite infection. This might reflect a *Leishmania* parasite adaptive strategy to maintain its transmission cycle in nature; such persistence can lead to disease reactivation; however, it could also contribute to the maintenance of a lifelong immunity against reinfection [38,225].

6.4.1.3 Anti-*Leishmania *immunity in different* Leishmania *species and hosts The fact that resistant inbreed strains of mice (self-resolution of lesions) develop a Th1 response, whereas susceptible strains (progressive nonhealing lesions) develop a Th2 response upon experimental infection by *L. major* provides an exquisite demonstration that Th1 and Th2 subsets can influence the course of disease toward opposite poles [145,152,191,202,321] (Fig. 6.12). In humans, (i) the observation of a strong humoral response (characterized by high anti-*Leishmania* antibody titers) during the course of disease; (ii) the fact that a delayed-type hypersensitivity (DTH; detected by the leishmanin skin test) response, which is a marker of cellular immunity, develops in cured patients; and (iii) the fact that DTH positivity is also detected in exposed healthy subjects (asymptomatic infection) are also compatible with the Th1/Th2 model of resistance/susceptibility established in mice infected by *L. major*.

However, in mice and other animal models, the importance of the Th1/Th2 dichotomy in determining the course of infection is less clear when animals are experimentally infected with other *Leishmania* species such as the "visceralizing" species of the *L. donovani* complex [28,197,228]. Similar to *L. major* infection, resistant mouse strains such as C57BL/6 develop a Th1 response with $CD4^+$ cells, producing IFN-γ and IL-2 during *L. donovani* or *L. infantum* infections, whereas susceptible strains (BALB/C) exhibit a decrease in IFN-γ production. In contrast to *L. major* infection, the IL-12 effect is delayed for 4 weeks after infection. Furthermore, susceptible strains lack Th2 immune response despite disease progression [172,224,228]. Thus, in "visceralizing" *Leishmania* infection, Th1 response is not suppressed by Th2 response, in contrast to *L. major* infection.

Mice have also been used to evaluate the immune response directed toward other New World *Leishmania* spp. (*L. mexicana*, *L. amazonensis*) causing cutaneous leishmaniasis and it revealed striking differences with the *L. major* model. Overall, although a protective response is quite clearly Th1 mediated in all species studied to date, it has become apparent that the relevant importance of the specific Th2 response in disease progression is clearly *Leishmania* species dependent. Old World species (*L. major* and *L. donovani*) diverged from the New World species some 40–80 million years ago. It is therefore not surprising that these different parasite species have developed different strategies to survive within different tissue sites and/or a different range of mammalian hosts [220].

It is worth noting that even susceptible mice experimentally infected, for example, by *L. donovani* complex parasites, are able to finally resolve infection spontaneously, which make them a better model of subclinical infection rather than progressive disease. In contrast, hamsters infected by *L. donovani* develop progressive disease that mimics human visceral leishmaniasis more closely [87,129,130,290,374]. Surprisingly, there are significant amounts of Th1 cytokines expressed in the spleen of hamsters, although little or substantial amount of IL-4 and IL-10 is present. Instead, susceptibility to *L. donovani* in hamster seems more to be mediated by a defect of NO production by iNOS in infected macrophages rather than the development of a Th2 response [224]. In dog also, a natural reservoir of *L. infantum*, studies done so far have not been able to clearly establish the existence of a Th1/Th2 dichotomy in susceptibility to canine leishmaniasis [8,68,243,263]. Although *resistance is associated with a Th1 response* (production of IL-2, TNF-α, and IFN-γ able to stimulate macrophage leishmanicidal activity [254,263,310], *susceptibility has not been shown to be associated with a Th2 response* [70,264].

6.4.1.4 The immune response in human leishmaniasis

In humans as in experimental models, different patterns of immunological response are observed according to the clinical manifestation and exposure to the different *Leishmania* species. Indeed, different T-cell type responses are observed among the different cutaneous forms of leishmaniasis. An absence of a Th1 response (rather than presence of Th2) is seen in diffuse cutaneous leishmaniasis, whereas patients with self-healing lesions develop a Th1 response [1,65,176,177]. High IFN-γ levels are also detected in chronic lesions and mucocutaneous leishmaniasis, which are rather characterized by a mixed Th1/Th2 response [19,69,204,223]. In the case of the immune response directed against visceralizing *Leishmania* spp., it was shown that peripheral blood mononuclear cells (PBMCs) from individuals with asymptomatic or subclinical infection respond to *Leishmania* antigens with proliferation and production of IL-2, IFN-γ, and IL-12. However, visceral leishmaniasis displays a cytokine profile of mixed Th1/Th2 characteristics such as IFN-γ along with IL-10 readily detected [128,170,179]. Furthermore, both Th1 and Th2 clones producing IFN-γ and IL-4 have been isolated from cured patients [178]. Thus, it has not been possible to clearly associate a Th2 polarity with nonhealing, systemic, or reactivation forms of leishmaniasis. Overall, IFN-γ-producing cells or mRNA remain readily detectable in patients with visceral leishmaniasis, PKDL [126], or chronic cutaneous leishmaniasis, and the opposing cytokine most commonly found in these clinical settings is not IL4 as in mice but IL-10 [10]. Interestingly, IL-10 is not a "pure" Th2 cytokine, as it can also be produced by alternatively activated macrophages and, as shown recently in mice, by Treg cells that also produce IFN-γ. In contrast to IL-4, which inhibits Th1 expansion, IL-10 action serves more to down-regulate the activation of macrophage microbicidal activity by IFN-γ producing cells. The role of Treg cells has been proposed to be both to control the severity of inflammation (which occurs within Th1-type responses and can be harmful to the host) and to promote long-term low parasite persistence in order to maintain a memory pool necessary to resist reinfection [38].

6.4.1.5 Conclusions

Although the control of infection is almost always associated with the development of a Th1 response, mechanisms promoting disease susceptibility are not yet fully understood and determinants other than the Th2 cell subset are likely involved depending on the parasite species. Although macrophages are the primary host cell for *Leishmania*, the role of these cells has not been well characterized either in disease prevention or in disease progression. The effector functions of macrophages have always been described in a T-cell-dependent manner and the fate of infected macrophages in the pre-T-cell phase is not well known. It is also obvious that the parasites modulate the macrophage in terms of their antigen-presenting and intracellular signaling capacity. In this regard, intramacrophagic interaction (which needs to be further explored) between host and parasite molecules could regulate the capacity of macrophage to respond to IFN-γ (possibly through the secretion of IL-10 or other mechanisms) and explain the progression of disease in the context of a Th1 response.

6.4.2 Host Genetic Factors in Resistance/Susceptibility to Leishmaniasis

As stated above, immunological studies in experimental models of infection and in endemic area populations have

associated certain clinical manifestations with qualitative or quantitative changes in the host-specific immune response to *Leishmania*. Although these studies identified several immunological components that are markers of disease versus asymptomatic or subclinical infections, these studies did not identify the primary effects causing the observed complex immunological phenotypes. Growing evidence from mouse and human studies suggests that they are in part related to the genetic make-up of the host. This raises the hope that the analysis of host genetic susceptibility will help in identifying these defects and in demonstrating the causal link between immunological phenotypes and clinical diseases.

6.4.2.1 Genetic studies in mouse The existence of genetic host factors involved in resistance/susceptibility was first suggested by the fact that genetically distinct inbreed strains of mice exhibited substantial differences in the infection outcome in experimental infection by a single strain of *Leishmania*. The possibility of intercrossing or backcrossing between resistant and sensitive inbreed strains of mice has made it possible to start unraveling the basis of genetic susceptibility to leishmaniasis in these experimental models of infection. The control of the early stages of *L. donovani* infection (innate immunity) is associated with a mutation in the transmembrane domain of *Nramp1* (Natural resistance-associated macrophage protein 1) [52,378]. This gene, now renamed *Sl11a1*, codes for a macrophage-restricted divalent cation transporter that is recruited to the phagosome upon phagocytosis [322]. Although the mechanisms by which this transporter limits the replication of intracellular pathogens is not yet fully understood, it could be through the alteration of the phagolysosome environment, especially iron concentration, which is critical for the generation of oxygen-free radicals [43,46,390]. Furthermore, late control of *L. donovani* infection in susceptible mouse strains, concomitant with the development of acquired immunity, has been associated with alleles at the MHC locus [44].

It is worth noting that genetic determinants of murine cutaneous leishmaniasis (*L. major*) map to different regions of the mouse genome and appear to be more complex because 10–15 loci were implicated in the control of diverse clinical or immunological phenotypes [34,134,199,284,285,288,379]. Although the causative genes in these regions remain to be identified, they pointed to interesting positional candidate genes: (i) a susceptibility locus encoding a number of Th2 cytokines on mouse chromosome 11 is syntenic with human 5q31-33, which was shown to influence infection levels by *Schistosoma mansoni* [207], *Plasmodium falciparum* [124,281], and susceptibility to asthma [208]; other loci include genes encoding (ii) other cytokines or cytokine receptors (*Ifng*, *Ifngr1*, etc.); (iii) macrophage effectors (*Nos2*); (iv) transcription factors (*Stat6*); and so on.

Analysis of genetic susceptibility (control of lesion growth) of mice to infection by *L. mexicana* (causing human cutaneous leishmaniasis), also revealed important differences with *L. major* because it was mapped to a single locus on mouse chromosome 4 [287]. On the contrary, in *L. mexicana* infections, visceralization seems to be influenced by the *Nramp1* and *H2* loci (MHC) [286], as in *L. donovani*, whereas with *L. major* this phenotype is controlled by different genes (which remain to be identified) located on chromosomes 2 and 11. In mice, susceptibility to disease caused by different *Leishmania* species thus appears to be regulated by multiple, distinct genetic loci; therefore, it is not surprising that the immune regulation of disease and healing to each species also differs.

Infection of BALB/c-susceptible and C57BL/6-resistant mice by *L. major* has been extensively used to study the immunological determinants of a Th2 versus Th1 response. This huge body of work led to the widely admitted idea that *Th1 is protective in leishmaniasis, whereas Th2 is associated with susceptibility*. However, it is not yet clear how the association occurs: *does the host control the parasite because it develops a Th1 response or is it because parasite multiplication is controlled that a Th1 response eventually develops*? Genetic dissection in mice of clinical, immunological, or infection phenotypes [198] will help answer this question. Interestingly, in C57BL/6 and BALB/c mice congenic for three *L. major* susceptibility loci, the cytokine profile (Th1/Th2) correlated with the parental genetic background (C57BL/6 or BALB/c) but not with disease severity [118].

Experimental infection in inbreed strains of mice has made it possible to characterize the host immune response directed against *Leishmania* parasites and to identify genetic determinants responsible for the differences in resistance versus susceptibility. In mice, resistance/susceptibility was found to be controlled by different sets of genes according to the parasite species involved. Moreover, experimental infection by a given parasite species was found to elicit different immune responses according to the animal model under scrutiny. The question now is to validate these experimental observations in humans living in endemic areas, because experimental conditions of infection in mice are very far from natural infection in which only a few promastigotes are delivered in the host derma along with *Phlebotomus* salivary antigens.

6.4.2.2 Human genetic factors involved in resistance/susceptibility Evidence is now emerging that *host genetic factors also influence the outcome of human infection by* Leishmania. Epidemiological studies have shown familial aggregation of clinical phenotypes that are consistent with the existence of inherited factors in susceptibility to CL and VL in humans [58,63,399]. Furthermore, some studies indicated that the distribution of disease phenotypes (CL or VL) in extended pedigrees living in endemic areas are statistically best explained by the segregation of *one or two major susceptibility loci* [3,256,324]. Together with the observation of profound ethnic differences in the ratio of asymptomatic to symptomatic infections [58,153], these epidemiological observations strongly suggest that human susceptibility to cutaneous or visceral leishmaniasis is mediated by host genetic factors.

6.4.2.3 The candidate gene approach Identification of genes or genetic loci in mice accounting for the differences in susceptibility/resistance among the different

inbreed strains of mice opened the way to a mouse-to-man strategy to identify human susceptibility/ resistance genes [45]. Thus, early attempts to identify genetic factors in human leishmaniasis have first focused on polymorphisms in candidate genes tested by means of either case–control studies (association) or family-based studies (transmission disequilibrium test [TdT] or linkage analysis). Associations have been reported between some HLA alleles and the diverse forms of cutaneous leishmaniasis (CL and MCL) [27,190,258]. In contradiction with the mouse model, however, all attempts to demonstrate such an association with visceral leishmaniasis – in Brazil [45,257], India [329], Sudan [57], and the Mediterranean area [222] – have failed, pointing out the existence of important differences between humans and mice. Polymorphisms in the *TNFA* and *TNFB* genes in the MHC class III region, which encode TNF-α and lymphotoxin-α, respectively, have been associated with mucocutaneous leishmaniasis [64] (some of the promoter polymorphisms such as the *TNFA*-308 SNP were shown to be associated with cerebral malaria [218] and/or to drive higher transcription of the gene [391]). Polymorphisms of the *TNFA* promoter were also examined in asymptomatic *L. chagasi* infection and patients with visceral leishmaniasis [171]. Interestingly, in this study, genetic association was observed with the development of *Leishmania*-specific cellular immunity rather than disease itself. This suggests that although *TNFA* polymorphisms may be a risk factor for VL, they cannot alone explain the development of disease.

6.4.2.4 Genetic control of visceral leishmaniasis in two populations of eastern sudan

Two recent studies have started to study the genetic control of visceral leishmaniasis in Eastern Sudan, an area endemic for *L. donovani* and where an upsurge of cases has occurred over the last decade. One of the studies was carried out during an outbreak (1995–2000) that caused infection in almost all inhabitants of a village of the Sudanese–Ethiopian border (Fig. 6.13). Though more than 90% of the villagers showed immunological evidence of infection, 25% developed visceral disease. Substantial differences in disease prevalence were observed between ethnic groups living in sympatry (the Haoussa and Fellata having a decreased risk of developing VL compared to the Aringa), and

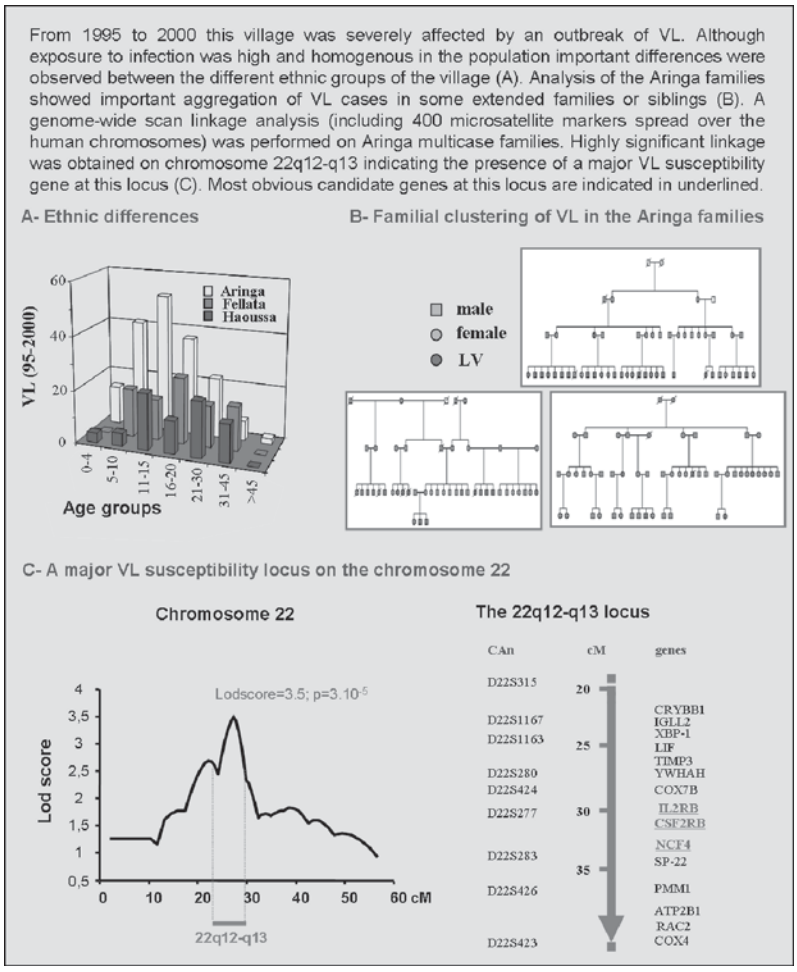

Fig. 6.13. The influence of host factors in an outbreak of VL in the village of Barbar El Fugarra (Eastern Sudan).

certain families among the Aringa ethnic group were more affected than others, suggesting the existence of a familial component in susceptibility to VL [57,58,115]. An attempt to demonstrate that this familial component was attributable to a major gene segregating in the Aringa pedigree was unsuccessful, indicating that the hypothesis of a single major locus is probably not tenable. However, a linkage study carried out on affected sib-pairs, a strategy that accommodates a multigenic control and scanning the entire genome, showed that one locus (chromosome 22q12, $p = 3.10^{-5}$) and possibly another one (chromosome 2q22-q23, $p = 0.0006$) controlled susceptibility to disease in the Aringa population [55]. The two loci identified by this study do not contain classically tested candidate genes in leishmaniasis and therefore allow the formulation of a new hypothesis on susceptibility/resistance genes important in human infection by *L. donovani*. Identification of these genes is now required to characterize the critical steps in the pathological process involved in this lethal disease. Several interesting genes are present at 22q12, such as *CSF2RB*, encoding the β chain of the granulocyte-macrophage stimulating factor (GM-CSF) receptor, and *NCF4*, encoding the soluble P40-PHOX subunit of the NADPH oxidase complex involved in the generation of superoxide in phagocytic cells. Another gene, *IL2RB*, encoding the IL-2 receptor β chain, could also be involved in susceptibility through the modulation the T-cell response by IL-2. However, it is not yet known if a single gene or several genes at the 22q12 locus contribute to the linkage observed in the frame of this study. It is interesting to note that two other genes, *NMI* (N-myc interactor; OMIM 603525) and *STAM2* (signal transducing adapter molecule 2; OMIM 606244), located in the 2q22-q23 region, are also coding proteins, involved in modulating signal transduction downstream of the IL-2 receptor, reinforcing the hypothesis that the IL-2 signaling pathway plays an important role in determining susceptibility to VL. Furthermore, the study carried out in this village population also suggests that host genetics might play an important role in outbreaks determining to a large extent which subjects are at risk of severe disease. The reduction in the number of susceptible subjects, due to patient death and to the induction of protective immunity in cured patients, has probably played a significant role in the termination of the outbreak. Furthermore, the arrival of genetically susceptible immigrants and children born after the outbreak from cured susceptible parents could create the conditions of a new outbreak and account for the periodic occurrence of KA outbreaks in this region of Sudan. In this regard, the Haoussa and Fellata, who first settled in this village in the 1940s, had experienced an outbreak of KA before the one documented in this study and were less affected by the present outbreak than the Aringa who had immigrated to the village more recently.

The other study was carried out in an area located 70 km away from the area covered by the above-described study and involved KA patients from the Massalit ethnic group (a group closely related to the Aringa in that they originate from the same geographic area in Western Sudan and Chad). Only a candidate gene approach was reported from this study [47]. Suggestive linkage (Lodscore = 1.8, $p = 0.002$) and significant association with VL (TdT, $p = 0.008$) were obtained with an intragenic microsatellite of the *IL4* gene in the 5q31-33 region (syntenic with the locus controlling visceralization of *L. major* on mouse chromosome 11). These results are consistent with a functional polymorphism in the close vicinity of the *IL4* gene controlling susceptibility to VL in the Massalit tribe. Another microsatellite marker in the *IFNGR1* locus indicated a possible implication of this gene (Linkage: Lodscore = 0.73, $p = 0.035$; TdT, $p = 0.007$) in the development of PKDL, although a larger sample size is required to confirm this result [232]. Interestingly, the 5q31-33 region and the *IFNGR1* locus showed no linkage in the Aringa study [57]. The reason for this may be that (i) although the Aringa and Massalit are a closely related ethnic group, subtle genetic differences may exist between these two groups; or that (ii) the expression of genetic susceptibility at the population level will depend on the environment, that is, transmission intensity or parasite diversity. In this regard, we can note that the Aringa were submitted to particularly high transmission levels during the Barbar El Fugarra outbreak, which may have driven higher infection rates compared to the Massalit.

Both studies in Sudan tested linkage with *SL11A1* (*NRAMP1*) polymorphisms. Suggestive linkage (Lodscore = 1.29) was observed in the Aringa [57], linkage was confirmed in the Massalit tribe (Lodscore = 1.41), and involvement of the *NRAMP1* gene was further confirmed by transmission disequilibrium test analysis [233]. Thus, the gene encoding NRAMP1, or a closely linked gene, is involved in determining genetic susceptibility to VL. Given the large body of literature on the role of *NRAMP1* in both mouse and human susceptibility to diverse intracellular pathogens [46], these results are consistent with a role of NRAMP1 in susceptibility to VL. However, given the low strength of genetic linkage or association at this locus, its role in determining the infection outcome in humans is minor compared to the strong effect observed in mice.

6.4.2.5 Conclusions Overall studies in mice and in populations living in endemic areas suggest that the risk of clinical leishmaniasis (CL, MCL, and VL) is markedly increased by allelic variants at specific genetic loci. Loci associated or linked with human leishmaniasis are summarized in Table 6.1. Some loci seem specific to particular clinical manifestations such as HLA/cutaneous leishmaniasis (CL, MCL) or *NRAMP1*/VL, whereas others may act across different clinical presentations (cytokines and cytokine receptor genes). Additional work in distinct populations is required to definitively establish the involvement of certain of these genes in susceptibility/resistance to leishmaniasis. It is worth noting that different ethnic groups display different susceptibilities to disease, which seems a common feature and should be carefully taken into account in order to avoid type I errors (false-positive associations) due to population admixture.

TABLE 6.1. Unraveling Human Genetic Susceptibility to Leishmaniasis: a Review of the Literature

Country	Population	Phenotype (*Leishmania* sp.)	Locus	Candidate genes or alleles	Genetic association/ linkage	References
Candidate gene approach						
French Guyana	Hmong refugees	**CL** (*L. guyanensis*)	6p21	HLA	+	[27]
Venezuela		**CL** (*L. braziliensis*)	6p21	HLA	+	[190]
Brazil		**MCL** (*L. braziliensis*)	6p21	HLA	+	[258]
Brazil, India		*VL (L. donovani complex)*	*6p21*	*HLA*	−	*[47,56,222, 257,329]*
Sudan, Mediterranea						
Venezuela		**MCL** (*L. braziliensis*)	6p21	TNFA/TNFB	+	[64]
Brazil		**DTH+** (*L. chagasi*)	6p21	TNFA	+	[171]
Sudan	Aringa, Massalit	**VL** (*L. donovani*)	2q35	NRAMP1	+	[397,398]
	Massalit	**VL** (*L. donovani*)	5q31	IL3, IL4, IL5, IL9, IL12p40, IRF1, CSF2, CSF1R	+	[397,398]
	Aringa	*VL* (*L. donovani*)	*5q31*	*IL3, IL4, IL5, IL9, IL12p40, IRF1, CSF2, CSF1R*	−	*[56]*
	Massalit	**PKDL** (*L. donovani*)	6q23	IFNGR1	+	[232]
Genome-wide approach						
Sudan	Aringa	**VL** (*L. donovani*)	22q12	IL2RB, CSF2RB, NCF4(P40-phox)	++	[55]
			2q23-q24	NMI, STAM2	+	[55]

Results of human genetic studies carried out on diverse populations of the world exposed to diverse *Leishmania* species. Studies are ordered by genetic loci and approaches (candidate gene testing vs. genome-wide analysis). Positive results are given in bold and negative results are in italics.

As evidenced by the genome-wide scan described above, the candidate gene approach, relying heavily on the results obtained in experimental models, is likely to miss important genes specific to natural infection in humans. Other such global approaches on different populations (linkage genome-wide analysis, microarray transcriptom analysis, etc.) in which no hypotheses are made on the genes involved will be required in the future and constitute the first step toward their identification. High-throughput genotyping methods are now becoming available that make it possible to characterize individual genotypes at thousands of polymorphic sites in the human genome in a very short time. Although the related cost of such approaches are today too high to be used extensively (especially for the limited budgets in infectious disease research), we hope that these will become increasingly available in the coming years and help analysis of human susceptibility to leishmaniasis on a global scale. However, the possibility of genotyping hundreds of subjects for thousands of genetic markers should not override the fact that the selection of the population sample to be used in the analysis is the most critical step.

As we have stated earlier in this chapter, not only genetic factors from the host are involved in determining susceptibility/resistance to leishmaniasis. The possibility of integrating other risk factors (from the environment or the parasite) at the individual level should thus help select leishmaniasis patients for whom genetic susceptibility is the most likely and in turn increase the power to detect and identify host genetic effects. The asymptomatic phenotype is also probably quite heterogeneous, with subjects being asymptomatic carriers, those developing unnoticed subclinical disease, and others clearing infection totally. Characterization of the quality of the immune response, demonstration and quantification of the parasite in these "asymptomatic" individuals could help split this complex phenotype into more homogeneous sub-phenotypes for genetic analysis.

In summary, genetics of the host could provide critical information for the discovery of key steps in the pathogenesis of *Leishmania* infections and allow the identification of new targets (targeted on the host response rather than the parasite) for chemotherapy and vaccination. We can also hope that genetic studies will allow the identification of subjects at high risk of severe disease. Such subjects could benefit from targeted prophylactic measures; they will also be evaluated carefully in drug and vaccine trials, as different proportions of susceptible/resistant subjects in the vaccinated or placebo group could be important confounding factors in the analysis.

Fig. 6.14. Transmission of kala-azar in Bihar, India.

6.5 THE NEED FOR AN INTEGRATED APPROACH: THE KALA-AZAR EXAMPLE IN INDIA

In all the previous sections, we have detailed the different known factors involved in leishmaniasis. In this last section, we will focus on the need for an integrated approach considering parasite, vector, and host involvement with the example of Indian VL.

In India, millions are at risk; the state of Bihar accounts for nearly one-fifth of worldwide cases (Fig. 6.14). The current episode of leishmaniasis in India is unique: the disease started in the early 1970s, and for more than 30 years there has been incessant transmission spreading in all directions. There have been efforts to control the disease, mostly knee-jerk reactions, and this has hardly had an impact on transmission. Affected populations (Fig. 6.15) are among the poorest in the world and are not much informed on existing preventive measures. Furthermore, misuse of the first-line drugs in these communities is widespread [342], and the lack of response to pentavalent antimonials has been increasing sharply over the last few years in India, up to more than 50% of the patients in the hyperendemic areas of Bihar [201,328,352].

In these hyperendemic areas, it is now well established that most exposed individuals are asymptomatic [22,336,399]. A multidisciplinary approach, combining parasite, host, and vector studies, could help (i) to understand why in an endemic area in Bihar different clinical outcomes (asymptomatic/paucisymptomatic vs severe visceral disease) result from infection by *L. donovani* and (ii) to identify factors determining resistance or susceptibility to the disease. The integrated analysis of parasite genetics, parasite virulence factors, host immune responses, host genetics, as well as socioeconomic and environmental risk factors will provide a better understanding of the interplay between these different factors and the risk of

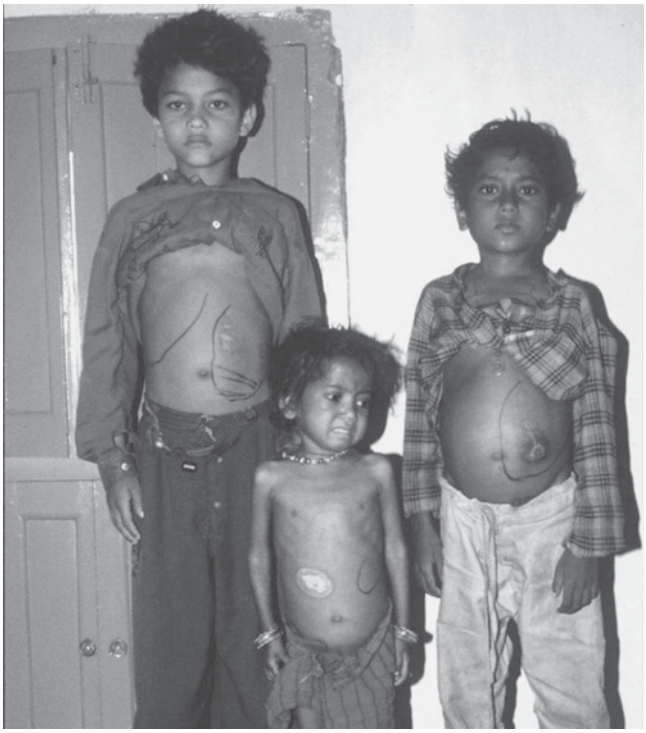

Fig. 6.15. Children with Indian kala-azar with burn marks on the abdomen in an effort to cure by traditional healers (all rights reserved).

developing VL, the critical biological pathways involved in host resistance or susceptibility to VL, and therefore help orient new therapeutic or vaccine strategies.

Vector control should include not only insecticide spraying but also household vector control measures such as insecticide-impregnated bed nets and curtains, sanitation improvement, and elimination of the sand fly breeding sites. There is an absolute lack of awareness regarding the etiology, transmission, and factors favoring growth of sand flies. Thus, the piecemeal efforts are not likely to succeed. A well-coordinated effort with a combined IEC (information, education, and communication) approach, multipronged vector-control measures, including elimination of breeding sites, personal protection, and insecticide spraying, early diagnosis and effective treatment accessible to all, either free or at a subsidized cost, can only make a favorable impact on transmission.

Nevertheless, to be efficient, these measures require an accurate knowledge of the vector's ecology (geographical distribution, species involved, habitat, transmission rate).

Lack of a vaccine is one of the strongest drawbacks in controlling VL in India and other endemic regions. Intensive efforts toward vaccine development with fast-track clinical development and approval are crucial. Exact immunological aberration in VL has yet to be unraveled, and continued research in human VL can improve the understanding of the disease and provide important clues toward immunotherapy as well as vaccine development. Concerning a VL vaccine, evaluation of the different players' involvement is a critical step in a phase III vaccine evaluation trial. Indeed, inclusion in the vaccinated and placebo groups of different proportions of resistant and susceptible subjects could seriously impair the results of the study. Concerning this last point, a multidisciplinary approach would help in identyfing resistant and susceptible populations in a given endemic area. Moreover, the study of the vaccine antigen-specific antibodies and the cellular immune responses induced *ex vivo* in T-cell stimulation assays in VL patients before and after treatment and in asymptomatic subjects will provide the identification of a number of immunologic parameters in subjects exhibiting patterns of progressive disease or apparent resistance. This is a fundamental prerequisite to identifying the interactions between various cell types that are involved both in processing and effector responses. This would facilitate our understanding of the disease and help in the design of a vaccine against VL.

6.6 CONCLUSION

In this chapter, we attempted to demonstrate the multifactorial aspect of leishmaniasis. The exact involvement of hosts, vectors and parasites, and interactions among them in the outcome of the disease remains unknown. Although we know that all of them have an impact on the manifestation of the disease: (i) there is a strong vector–parasite specificity; (ii) *Leishmania* species are statistically associated with certain clinical forms and some factors have been described as associated with clinical diversity; and (iii) the risk of leishmaniasis is markedly increased by allelic variants at specific host genetic loci – *it is necessary to study all of these factors in a population of a single focus and it is especially important to cross the results*. The integrated analysis on the same subjects (epidemiological, parasitological, immunological, and genetic studies) would therefore provide a clear picture of the interplay between environmental, parasitic, and host factors in the development of the disease. For example, in the case of the Indian focus, an integrated approach could help us to better understand (i) the increasingly worrying problem of drug resistance (Is it due evolution of the host-parasite system? What are the biochemical mechanisms involved?) and (ii) asymptomatic carriers (Are these people infected by a "particular" parasite and/or are they able to contain the infection by themselves? In both cases, what are the molecular processes involved?

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ABBREVIATIONS

ACL:	Anthroponotic cutaneous leishmaniasis
AIDS:	Acquired Immune Deficiency Syndrome
ATP:	Adenosine triphosphate
AVL:	Anthroponotic visceral leishmaniasis
CL:	Cutaneous leishmaniasis
CM-DM:	Canine monocyte-derived macrophages
CP:	Cysteine protease
DAT:	Direct antigenemia test
DNA:	Deoxyribonucleic acid
DTH:	Delayed-type hypersensitivity
ELISA:	Enzyme-linked immunosorbent assay
ESA:	Excreted-secreted antigen
fPPG:	Filamentous proteophosphoglycan
GM-CSF:	Granulocyte-macrophage stimulating factor
GP63:	Glycoprotein 63
GPI:	Glycosylphosphatidylinositol
HAART:	Highly Active Antiretroviral Therapy
HIV:	Human immunodeficiency virus
HLA:	Human leukocyte antigen
IFAT:	Immunofluorescence antibody detection test
IFN:	Interferon
Ig:	Immunoglobulin
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
KA:	Kala-azar
LPG:	Lipophosphoglycan
MCL:	Mucocutaneous leishmaniasis
MHC:	Major histocompatibility complex

MLEE: Multilocus enzyme electrophoresis
mRNA: Messenger ribonucleic acid
NADPH: Nicotinamide adenine dinucleotide phosphate reduced
NK: Natural killer
NMI: N-myc interactor
NO: Nitric oxyde
Nramp: Natural resistance-associated macrophage protein
PBMC: Peripheral blood mononuclear cell
PCR: Polymerase chain reaction
PFGE: Pulse field gel electrophoresis
PHOX: Phagocyte oxidase
PKDL: Post-kala-azar leishmaniasis
PM: Peritrophic matrix
PSA: Protein surface antigen
PSG: Promastigote secretory gel
RAPD: Random amplified polymorphic DNA
RFLP: Restriction fragment length polymorphism
Sb^V: Stibogluconate (pentavalent antimonials)
SNP: Single nucleotide polymorphism
STAM: Signal transducing adapter molecule
TdT: Transmission disequilibrium test
TGF: Transforming growth factor
Treg: Regulatory T cells
Th: T helper cells
TNF: Tumor necrosis factor
VL: Visceral leishmaniasis
ZCL: Zoonotic cutaneous leishmaniasis
ZVL: Zoonotic visceral leishmaniasis

GLOSSARY

Acquired immune response: Immunity mediated by lymphocytes and characterized by antigen specificity and memory. It is a specific, inducible immune response to pathogens.

Affected sib-pair study: This is the most familiar form of nonparametric linkage analysis. This is observed if affected sibling pairs inherit the same marker allele from their parents more frequently than would be expected by chance.

Allele: An allele is a variant of a single gene, inherited at a particular genetic locus; it is a particular sequence of nucleotides, coding for messenger RNA.

Antibodies: Any of numerous molecules of immunoglobulin superfamily produced by the B cells as a primary immune defense in response to specific proteins (antigens).

Antigen: Any substance recognized by the body as being foreign, that stimulates the production of antibodies. These antigens produce an immune response by the organism in response to their presence.

Biological concept of species: It defines species in terms of interbreeding. For instance, Ernst Mayr defined a species as follows: "species are groups of interbreeding natural populations that are reproductively isolated from other such groups." The biological species concept explains why the members of a species resemble one another, that is, form phenetic clusters, and differ from other species.

Case–control studies: They are based on the comparison of genotypes or allele frequencies between affected cases and unaffected controls groups.

Chemotherapy: The treatment of disease by means of chemicals that have a specific toxic effect on the disease-producing microorganisms (e.g., antibiotics) or that selectively destroy cancerous tissue (anticancer therapy).

Clone, *clonal*, *clonality*: From a genetic point of view, this term refers to all cases in which the daughter cells are genetically identical to the parental cell, whatever the actual mating system.

Cytokines: Small proteins or biological factors (in the range of 8-30 kDa) that are released by cells and have specific effects on cell–cell interaction, communication, and behavior of other cells.

Endemic disease: Present or usually prevalent in a population or geographical area at all times.

Epidemiology: The study of the distribution and determinants of health-related states and events in populations and the control of health problems.

Genetic association: It is related to observing if a particular gene polymorphism is statistically associated with disease, it can be carried out by different methods (case–control studies or family-based association tests).

Genetic locus: The site in a linkage map or on a chromosome where the gene for a particular trait is located. Any one of the alleles of a gene may be present at this site.

Glycoconjugates: Carbohydrates covalently linked to a nonsugar moiety (lipids or proteins). The major glycoconjugates are glycoproteins, glycopeptides, peptidoglycans, glycolipids, and lipopolysaccharides.

Immune response: Alteration in the reactivity of an organism immune system in response to an antigen; in vertebrates, this may involve antibody production, induction of cell-mediated immunity, complement activation or development of immunological tolerance.

Immunization: The act of inducing antibody formation leading to immunity.

Immunogenic: Producing immunity, evoking an immune response.

Immunosuppression: This occurs when T and/or B lymphocytes are depleted in size or suppressed in their reactivity, expansion, or differentiation.

Incidence: The frequency of new infections during a designated time period expressed.

Innate immunity: Early nonspecific immune response to pathogens. Also called natural immunity, it is immediate first line of defense, including the complement system, natural killer cells and phagocytic cells (e.g., macrophages, dendritic cells).

Kinetoplast: Mass of mitochondrial DNA, usually adjacent to the flagellar basal body, in flagellate protozoa (these microorganisms belong to the group of kinetoplastids (Kinetoplastida order).

Leishmanization: An ancient practice of immunization to protect against infection to *Leishmania* by inoculating live parasites.

Linkage analysis: A mean for determining the localization in the genome of an unknown susceptibility gene with respect to positionally known genetic markers. This is based on the tendency for closely positioned sequenced to be inherited together.

Lodscore: It represents the intensity of linkage between two markers on the genome (the logarithm of the likelihood ratio for the odds in favor of linkage over no linkage). It provides a statistical test of the null hypothesis of free recombination (no linkage) over the alternative hypothesis of linkage.

Lutzomyia: A genus of New World sand flies or bloodsucking midges (family Psychodidae) that serve as vectors of leishmaniasis and Oroyo fever; formerly combined with the Old World sand fly genus *Phlebotomus*.

Major histocompatibility complex (*MHC*): The set of gene loci specifying major histocompatibility antigens. The MHC molecules display antigenic peptides to T lymphocyte receptors and initiate the specific immunity.

Metacyclogenesis: Process by which noninfective procyclic promastigotes are transformed into metacyclic promastigotes, the infectious form. This process is characterized by morphological changes of the parasite and also biochemical transformations.

Multilocus Enzyme Electrophoresis (*MLEE*): Protein extracts from given samples, for example various pathogen stocks, are separated by electrophoresis. The gel is then subjected to a histochemical reaction involving the specific substrate of a given enzyme, and the zone of activity of this enzyme is specifically stained. The same enzyme from different samples may migrate at different rates. These different electrophoretic forms of the same enzyme are referred to as isoenzymes or isozymes.

Pathogenicity: The ability of a pathogen to inflict damage on the host.

Pedigree: A multigenerational family health history diagrammed with a set of international symbols to indicate the individuals in the family, their relationships to one another, those with a disease, and so on.

Phagocytosis: Phagocytosis involves the ingestion and digestion by phagocyte cells of microorganisms, insoluble particles, damaged or dead host cells, cell debris or activated clotting factors. The principal phagocytes include the neutrophils and monocytes (types of white blood cells).

Phenotype: The observable characteristics of an organism, the expression of gene alleles (genotype) as an observable physical or biochemical trait. It is the result from interaction between the genotype and the environment.

Phlebotomus: A genus of psychodidae that functions as the vector of a number of pathogenic organisms, including *Leishmania*.

Polymerase chain reaction (*PCR*): A technique to amplify a specific region of double-stranded DNA. An excess of two amplimers, oligonucleotide primers complementary to two sequences that flank the region to be amplified, are annealed to denatured DNA and subsequently elongated, usually by a heat-stable DNA polymerase from Thermus aquaticus (Taq polymerase).

Prevalence: The proportion of individuals in a population having a disease.

Promastigote: Term now generally used instead of "leptomonad" or "leptomonad stage," to avoid confusion with the flagellate genus *Leptomonas*. It denotes the flagellate stage of a trypanosomatid protozoan in which the flagellum arises from a kinetoplast in front of the nucleus and emerges from the anterior end of the organism; usually an extracellular phase, as in the insect intermediate host (or in culture) of *Leishmania* parasites.

Prophylaxis: The administration of chemicals or drugs to members of a community to reduce the number of carriers of a disease and to prevent others contracting the disease.

Reservoir host: A reservoir is the source of an infecting microorganism. It serves as a source from which other individuals can be infected. For example, a zoonosis is a communicable disease that is transmitted from a nonhuman animal (*reservoir*) to a human.

Sib-pairs: See affected sib-pair study.

Taxonomy: The theory and practice of biological classification. The theories and techniques of naming, describing, and classifying organisms, the study of the relationships of taxa, including positional changes that do not involve changes in the names of taxa.

Transmission disequilibrium Test (*TdT*): It is a family-based association test. In this case, only cases and their parents are included in the analysis. The TdT is used to look for bias in transmission of alleles from heterozygous parents to affected offspring (different from 0.5 if there is association).

Vector: An agent, usually an animal or an insect, that transmits a pathogen form one host to another.

Zymodeme: Regroups all the *Leishmania* strains that have the same MLEE patterns for all the loci.

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CHAPTER 7

Epidemics of Plant Diseases: Mechanisms, Dynamics and Management

Serge Savary

INRA, Centre de Bordeaux, UMR Santé Végétale, France

7.1 BOTANICAL EPIDEMIOLOGY

Botanical epidemiology deals with interacting populations of host plants and plant pathogens [71]. This field of science is at the interface of microbiology, plant physiology, ecology, environmental physics, genetics, statistics, economics, and applied mathematics. The purpose of this section is to provide the reader with an overview of concepts, methods, and definitions that shape both research and applications in botanical epidemiology, as a discipline. In particular, the field emphasizes host–pathogen interactions at the populational level; these interactions depend on the physical and biological environment; and much of these, at least in agroecosystems, depend on man-made management practices. Figure 7.1 thus illustrates the paradigm where modern botanical epidemiology evolves [91]: a pathogen population (B), a plant host population (P), their environment (E), and man's actions (M). This figure provides the context where the field of botanical epidemiology evolves, and also why it leads to applications: man's actions imply management, or mismanagement, of what has been called a pathosystem [56]. This framework of thinking invites consideration of populations, rather than individuals, of interactions, rather than main effects, and of the dynamics of a system, rather than its status at a given point of time. All these are best addressed through different kinds of modeling methods.

A classical example of man's influence on plant diseases is that of nitrogen fertilization on rice diseases [52]. Although nitrogen is one important input to achieve higher attainable yield, high nitrogen application favors profuse growth of a darker, more humid canopy, and so favors some diseases, such as blast and sheath blight (both caused by fungi, *Magnaporthe grisea* and *Rhizoctonia solani*, respectively). However, lack of nitrogen, inducing poor growth and lower attainable crop yields, often favors other diseases, including brown spot (*Cochliobolus miyabaenus*). Contrary to a common misconception, there are diseases for poorly or well-tended crop stands, for healthy or unhealthy crops, and for rich or poor farmers [88].

One important remark must be made here: botanical epidemiology was borne and exists as a discipline because of the effects epidemics may have on cultivated plant communities, that is, fields, and for the need to understand, and so, manage epidemics in crops where they can cause disastrous losses [39]. This is briefly addressed later on in this chapter. However, botanical epidemiology also does address epidemics in spontaneous plant communities (see, e.g. [4,17, 28, 41]). A key difference between the two types of plant communities is that a crop typically consists of plants of the same age, that is, cohorts of individuals. When dealing with cultivated crops, therefore, botanical epidemiology profoundly differs from medical or animal epidemiology, as well as from epidemiology in spontaneous plant populations, where the host population generally consists of several age cohorts.

Pathogens are major drivers of the diversity of species, including plants, in the biosphere (see, e.g. [3 ,76]). They also contribute to the diversity of crop management and practices in the agricultural world [58]. Although botanical epidemiology primarily deals with practical, and important, issues that pertain to food security and food safety, it rests on scientific principles. Many of these have been derived from studies in cultivated plant stands, where experiments involving artificial infection, spatio-temporal analysis of disease progress, and the study of the diseased population performances have been comparatively easy to conduct.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

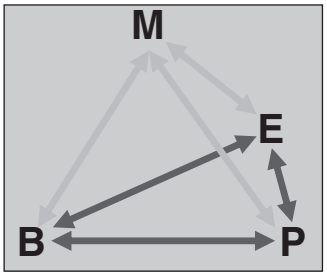

Fig. 7.1. The epidemiological tetrahedron and the four components of botanical epidemics: a pathogen population (B); a host plant population (P); their environment (E); and the over-arching effect of man (e.g., through cropping practices in agroecosystems).

7.2 PHENOMENOLOGY OF BOTANICAL EPIDEMICS

Botanical epidemics may take a number of shapes over time [31], although this diversity may be explained by only a few components: the amount of primary inoculum of the pathogen, the existence and number of successive cycles during the life cycle of the host plant population, and the existence of a limit to disease progress. Figure 7.2 illustrates typical shapes of epidemics. Epidemics of the type in Figure 7.2a are seldom found: rarely are epidemics unlimited in their spread, unless the host plant cycle is short enough, and successive, concatenated disease cycles are numerous enough in a short lapse of time. More frequent is the type in Figure 7.2b, where no secondary spread of the disease occurs during the host's cycle. Figure 7.2c exemplifies the prototype of epidemics that have been subject to the bulk of epidemiological research, with successive, concatenated cycles multiplying over time the disease from an initial amount of primary infections up to a limit set by the carrying capacity of the host population. Much of the early literature has been grounded on these shapes, especially the typical polycyclic epidemics of type 2c, providing the basis of the seminal work by Van der Plank [71, 72]. Shapes may be misleading, however, and Pfender [55] showed that phenomena must not be disconnected from their underlying mechanisms. In particular, many epidemics of type 2b, conventionally termed "monocyclic," may in reality hide a number of secondary spread events. Such is the case of, for example, many soilborne diseases, whose epidemic shapes reflect a restricted access of the pathogen to host tissues, and environmental effects.

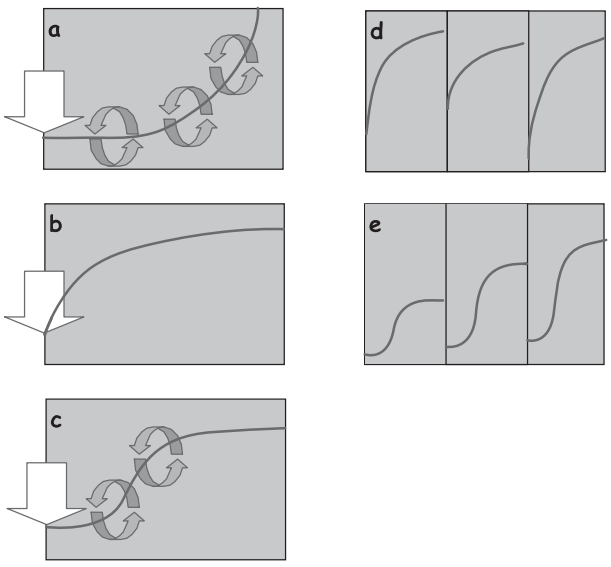

Fig. 7.2. Botanical epidemics as temporal phenomena. Large open arrow: initial inoculum (x_0); circling arrows: recurrent disease cycle during the host plant life cycle. (a – c): Epidemics in the course of a single host plant growing season: (a) exponential (polycyclic) epidemics; (b) monomolecular epidemic; (c) logistic (polycyclic) epidemic. (d, e) Examples of botanical epidemics concatenated over successive host plant cycles (polyetic epidemics): (d) polyetic monomolecular epidemics; and (e) polyetic polycyclic epidemics.

One important field where botanical epidemiologists have been investing time and efforts is the measurement of disease in a host population. This cannot be discussed here with any detail, and the reader is referred to James and Teng [22], Kranz [32], and Nutter et al. [51] for further information. At the center of disease assessment is the concept of lesion, which may, in botanical epidemiology, refer to different scales. Plant pathogens vary in their ability to invade host tissues, from locally to systemically. A lesion refers to the biological unit that enables the pathogen to multiply and spread. It thus may be an individual corn plant infected by the maize streak virus, a tomato root infected by a soilborne Oomycete such as *Pythium* sp., or an individual pustule on an oat leaf caused by *Puccinia coronata*. Being the source of possible further spread of disease, lesions are the epidemiological basis for measuring the amount of disease in a host population.

Several models have been developed to describe the temporal structure of epidemics. Most express the speed of epidemics, with the shape [36]:

$$\frac{dx}{dt} = g\ f(x)h(x), \quad \text{dimension} : [T^{-1}]$$

where x is the amount of disease in the host population (commonly, the fraction of host tissue diseased, i.e., disease severity, dimension [1]), t is the time (dimension [T]), g (often, a constant) is the apparent rate of disease (severity) increase, $f(x)$ represents the re-mobilization of inoculum from infected tissues, and $h(x)$ represents the consequences of the disease being present in the host population, especially in terms of tissues that are still healthy, and thus available to new infections.

Three main types of equations have conventionally been used, which correspond to the disease time–progress curves of Figure 7.2. A fourth type is indicated, which initiated important developments, from both the scientific and practical standpoints.

Kinetic type	$f(x)$	$h(x)$
Exponential	x	1
Monomolecular	1	$(1 - x)$
Polycyclic (logistic)	x	$(1 - x)$
Paralogistic	Infectious tissues at t	Healthy tissues at t

These equations have found many applications: they do encapsulate (very simple) hypotheses on the underlying mechanics of disease progress in time, are simple to manipulate, and lead to simple calculation of apparent rates of disease increase:

$$r = \frac{1}{t}\ln\frac{x}{x_0},\ \text{dimension}:[\mathrm{T}^{-1}];$$

$$r = \frac{1}{t_2 - t_1}(\ln x_2 - \ln x_1),\quad \text{dimension}:[\mathrm{T}^{-1}];$$

$$\text{and}\ r = \frac{1}{t_2 - t_1}\ln\frac{x_2}{1-x_2} - \ln\frac{x_1}{1-x_1},\ \text{dimension}:[\mathrm{T}^{-1}];$$

for the exponential, monomolecular, and logistic models, respectively.

Such calculations have proven extremely practical tools in comparing epidemics and measuring the efficiency of disease management, whether through increased host plant resistance, chemical control, or crop management. One widespread application of the logistic model in "polycyclic" epidemics has led to the conventional distinction of three phases in the development of diseases in cultivated fields. In a first phase, the correction coefficient for healthy tissue availability, $h(x) = 1 - x$ is close to 1, and the epidemic may be correctly described by an exponential equation. A typical limit to this phase is reached when x approaches 0.05. A second phase takes place when $0.05 < x < 0.5$, that is, until the inflection point of disease progress curve is reached. The terminal phase, $x > 0.5$, corresponds to disease severity tapering off. In many cases, and for several reasons [24], disease severity does not reach the carrying capacity, and $x < 1$. Distinguishing these three phases may seem oversimplifying the reality and restricting it to a limited number of cases. It nevertheless has proven very useful in the early stages when botanical epidemiology was put into practice, particularly for tactical control of foliar diseases of annual crops: the first phase is a vital one, when chemical action, if necessary, will be the most efficient. Efficiency of chemical control rapidly drops in the second phase, where injury is caused to the crop, which may translate in reduced performances. In the third phase, tactical decisions such as chemical applications are useless. Interestingly, the average (visual) detection level of disease occurring in the field lies between the first and the second phase. When farmers need to rely on tactical decisions to control diseases in their field, the window of time when action may alter the course of an epidemic, and possibly change the outcome of a cropping season, is extremely narrow [91]. Today, interest in tactical decision is progressively drifting away (but see the last section of this chapter); strategic management decisions, either short term (choice of cropping practices, of resistant cultivars) or long term (choice of cropping regimen, research prioritization at a regional scale), are becoming important arenas where epidemiological research is contributing [92].

Several models have actually been successfully used to describe epidemics. Three additional equations are given below, which are discussed in more detail in Madden [36], and Campbell and Madden [6].

Kinetic	Equation	$f(x)$	$h(x)$	Linearized form	Remarks
Gompertz	$dx/dt = r\,x\,(\ln(1) - \ln(x))$	x	$(\ln(1) - \ln(x))$	$-\ln(-\ln(x)) = -\ln(B) + rt$	Initial epidemic faster than the conventional logistic. Inflexion point at $x = 1/e$
Bertalanffy–Richards	$dx/dt = r(x/(1-m))(1 - x^{(1-m)})$	$(x/(1-m))$	$(1 - x^{(1-m)})$	$\ln(1/X^{(1-m)} - 1) = -\ln(B) + rt$	m is a shape parameter. For $m = 0$, this amounts to the monomolecular model. When m tends to 1, this amounts to Gompertz. When $m = 2$, this amounts to the conventional logistic
Weibull	$dx/dt = (c/b)[\ln(1/(1-x))]^{(c-1)/c}(1-x)$	$(c/b)[\ln(1/(1-x))]^{(c-1)/c}\,1 - x$		$\{\ln[1/(1-x)]\}^{(1/c)} = t/b - a/b$	a is a position parameter (epidemic onset), b is a scale parameter (proportional to the inverse of disease increase), and c is a shape parameter of the disease progress curve

The simplicity of these models reflects a number of hypotheses, including (1) that two types of host tissues only are considered, healthy or diseased, (2) that r is constant over the course of an epidemic, (3) that all healthy tissues are equally exposed to infection, and (4) that the host population size does not vary during the course of an epidemic. A large amount of epidemiological research has dealt with these issues. Hypothesis (1) was addressed at an early stage by Van der Plank [71], and will briefly be discussed below. Hypothesis (2) refers to much of the idiosyncrasies of epidemics: each epidemic is unique, partly because of the biological and environmental (soil, climate) context where it develops; any change in host plant resistance (e.g., through aging), any variation in climate inevitably must translate in changes of r values. Hypothesis (3) links these equations with the spatial dimension of botanical epidemics: the probability of a healthy site of becoming diseased is not isotropically distributed; on the contrary, it depends on the spatial distribution of the host tissues, the spatial distribution of the attributes of the physical environment, and the spatial pattern of dispersal of the pathogen. This is a field of extremely active research today, and the reader is referred to several texts [6, 48, 60, 74]. Hypothesis (4) refers to change in host population size [6], especially host growth, and possibly host population reduction (e.g., disease-induced defoliation). This latter hypothesis has particular relevance in the context of cohorts of individuals constituting the host population, as is the case in (most) cultivated field populations.

Many botanical epidemics are polyetic, that is, the result of the concatenation of individual epidemic components occurring during successive cycles of a plant host population. Polyetic epidemics are commonplace in soilborne plant diseases, diseases in spontaneous or natural plant stands, whether annual or perennial, and diseases of perennial crops including fruit crops. One is then dealing with a higher level of integration, where (1) individual epidemic processes depend, to a varying extent, on the previous epidemic process in terms of primary inoculum (x_0), (2) these epidemic processes also depend on previous epidemic processes which may have affected the host population (size, growth, and structure), and (3) successive epidemic processes progressively shape the biological composition of both the host and pathogen populations (which is retained, possibly with a bottleneck effect in the primary inoculum generating each successive epidemic process). Polyetics are ancient phenomena, both in natural and agricultural ecosystems. They are comparatively new to botanical epidemiology, and represent a massive and exciting scientific challenge. Initial thoughts on this topic, with epidemics of order 0, 1, and 2, initiated as initial infection points leading to local disease foci (zero-order epidemic), to individual epidemic processes at the population scale, for example, a cultivated field (first-order epidemic), and concatenated epidemic processes, spreading from field to field and to continental scales in polyetic epidemics (second-order epidemics) may be found in Heesterbeek and Zadoks [20].

7.3 PROCESSES IN BOTANICAL EPIDEMICS

The terms "processes" and "concatenation" have been used several times in the previous section. They are used again here, but in a different context. Epidemics represent the usual level of integration [10] of interest for botanical epidemiologists. Below it are several levels: (i) the disease cycle (e.g., for a soilborne disease: inoculum mobilization, contact with the host tissue, infection, development of the pathogen within or onto the root system, secondary infections from root-to-root contacts, production of survival structures, and survival in the soil); (ii) processes governing individual stages of the cycle (e.g., for the germination of a fungal spore onto a leaf surface: leaf moisture and temperature, leaf age, phylloflora composition); (iii) the various substages which may be defined for each of these individual stages (e.g., the successive morphological steps of the germination of a rust urediniospore); down to (iv) processes governing individual substages at the finest level of biological integration, including, for example, the molecular dialogue between a susceptible potato root and a virulent cyst nematode, *Globoderra rostochiensis*.

Botanical epidemiology, just as medical epidemiology does, attempts to explain an epidemic not because of its shape as a phenomenon but because of the mechanisms that build it. Being used as the reference level of integration, an epidemic is seen as a system of its own (Fig. 7.1) and is explained from the level of integration immediately beneath it, the individual stages of the infection cycle.

Gaeümann [16] coined the concept of "infection chain" (Fig. 7.3) to represent the basis for understanding the underlying mechanics of botanical epidemics [30]. The concatenation of these elementary processes result in a cycle, a monocycle, which itself is the elementary component of an epidemic. Some (actually, few) epidemics consist of only one monocycle, and many others consist of few or several, concatenated monocycles. Figure 7.3 uses terms that pertain to a disease of the foliage caused by a fungus; other terms would have to be used for other diseases caused by quite different plant pathogens. Numerous environmental factors influencing individual stages could be linked to the chain (see section below), and their respective weight on the course of epidemics, indicated. Whichever the disease, each of its specific biological links and their particular behavior to environmental factors, the resulting diagram, an "ethograph" (C.A.J. Putter, personal communication) depicting the successive stages of an infectious cycle would retain the same overall shape. Drawing ethographs of plant diseases perhaps is mere basic biology. For botanical epidemiologists, it however serves the two important purposes of identifying where the chain might be broken, and so, an epidemic stopped, and of locating where environmental factors may play an important role in epidemics.

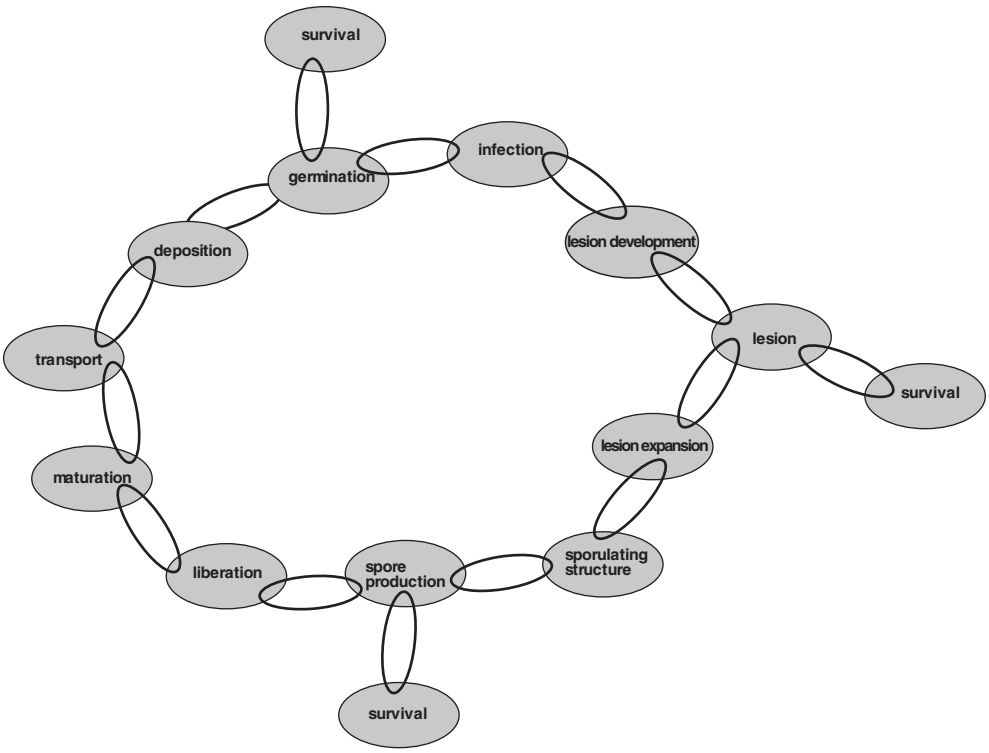

Fig. 7.3. The infection chain, after Gaeümann [16] and Kranz [30]. Epidemics are built from such monocycles. The terms used refer to a fungal pathogen of the aerial plant part of a host plant.

7.4 FACTORS INFLUENCING EPIDEMICS

The body of literature dealing with factors influencing epidemics is so large that only a very brief overview can be offered here. Analyzing environmental effects on botanical epidemics serves several purposes. One is to analyze and quantify how components of the monocycle react to changes in the environment, and so perhaps predict the behavior of epidemics [30]. Another is to determine whether an epidemic caused by a given pathogen, in a given host population, in a given environment, may occur [73]. A third is to determine whether the environment might be altered so that epidemics would be reduced: in cultivated host populations, crop management may then translate in disease management [53]. A fourth is the identification of specific intervention points in disease cycles [67] where targeted control action, including pesticide application, will have the strongest disease-reducing effect. Historically, the latter objective has attracted much effort.

The environment of epidemics is simultaneously physical, chemical, and biological, with two broad components in the latter, the physiological-genetic status of the host, and its microbiological environment. One convenient way of addressing the diversity of factors is to consider an electronic analogy [87], whereby the performance of a given stage in the monocycle (e.g., number of new pathogen propagules produced, number of successful virus transmissions per vector and per time unit, or latency period duration) is seen as responses to environmental stimuli [91]. Two groups of components of the monocycle may be considered with respect to the effects of the environment, a nondispersive and a dispersive phase ([34]; see also the last section of this chapter with an example of strategic management of a polyetic epidemic). The effects of environmental factors on the nondispersive and dispersive phases are thus briefly discussed here.

Soybean (*Glycine max*) is a major agricultural product of North America, where producers have to deal with a broad range of diseases caused by several viruses, airborne fungi, and soilborne pathogens. The above points are briefly illustrated here using this example. For many years, soybean leaf spot (*Cercospora kikuchii*) has been the main focus of research for its management. Stimulus-response studies have dealt with the ability of the causal fungus to grow and sporulate at varying light and temperature [7,8], infect the host at different development stage of plants [33], and its ability to survive and establish infections on leaf tissues that alternately are favorable (wet) or unfavorable (dry [61]). Web blight, caused by the soilborne fungus *Rhizoctonia solani*, is also an important soybean disease where summers are warm and humid, and crop canopies are dense and often wet. Spread of this type of disease results in conspicuous patches [80, 81], whose appearance and extension (several cm per day) strongly depend on canopy wetness, and thus, on crop density and management. Quantitative stimulus-response information was also gathered on another fungal disease, soybean rust (*Phakopsora pachyrhizi*),

which has long been feared in Northern America [82], including reaction types between different cultivars of the host and different isolates of the pathogen [45] and variation in host susceptibility with plant age [46]. Soybean rust may be dispersed over long distances by wind currents, and by late 2004, the disease was first observed in the southern part of the United States. Close surveillance is underway to monitor its overwintering and deploy different management options [83].

Dispersal of pathogens, that is, the dispersive phase of the monocycle, represents a field of its own in botanical epidemiology. Dispersal of pathogens, which may result in (but should not be confused with [25]) disease spread, depends on a large number of environmental factors, including the climatic environment of the host population stand (which affects the efficiency of the sources of inoculum and capture of inoculum by target host tissues [57]), the nature and structure of the inoculum source, and the physical characteristics of the host plant population (including the root or foliage system spatial structure and density). Introduction to this field may be found in Aylor [1], Zadoks and Schein [91], Jeger [25], and Campbell and Madden [6].

Again, only a very brief overview can be provided here, where emphasis is given to the very large body of empirical work that has been conducted. In an early review, Gregory [19] discussed the epidemiological implications of disease gradients whether caused by localized sources of inoculum or by environmental heterogeneity, using an empirical approach. The approach was later on reviewed by Fitt et al. [15], who analyzed 325 sets of observations describing the decrease with distance in deposition of airborne or splashborne fungal spores, or pollen, or in amounts of symptoms caused by fungi, bacteria or viruses. Two empirical models were compared:

The power law model $y = ax^{-b}$, or: $\ln(y) = \ln(a) - b \ln(x)$ and the exponential law model $y = c \exp(-dx)$, or $\ln(y) = \ln(c) - dx$ where y is the amount of disease (or deposition) and x is the distance from the source [19], and a, b, c, and d are parameters.

There is generally little difference between the two models in the goodness of fit to the data, although deposition gradients for spores borne in splash droplets are fitted better by exponential equations and gradients for fungi with airborne spores less than 10 m in diameter are fitted better by power law equations. The exponential model has the property that the modeled variable decreases by half as the distance from the source increases by a constant increment (the half-distance); this provides a measure of the gradient that is easier to visualize than the exponent of the power law model. According to the analytical review conducted by Fitt et al. [15], half-distances increase from 0.004–0.02 m for soilborne fungal pathogens, to 0.06–0.15 for splashborne bacteria and fungi, to 0.30–30 m for insectborne virus, bacteria, and fungi (with an extreme value of 4331 m for beet mosaic virus), and up to 0.7–130 m for airborne fungal spores (with an extreme value of $1.2\ 10^5$ m for *Puccinia graminis*, the stem rust pathogen). The exponential model is easier to incorporate into models of disease development than the power law model because the boundary condition at the source (the predicted amounts of propagules or of disease at the source) is finite rather than infinite.

Although both models can empirically be used to describe patterns of pathogen dispersal or of disease spread, they do not refer to similar underlying processes [15]. The exponential model implicitly refers to the extinction of a signal, with parameter b, away from the source of inoculum, whereas the power law model applies to dilution from turbulent diffusion as a cloud of particles moves away from the inoculum source. Both processes actually may occur simultaneously in a host population stand, and both models therefore may aptly be used to describe gradients in the same epidemic.

One important difference between the two models, however, is that the exponential equation predicts a much steeper gradient than the power law equation does at the tail of the gradient, with very important consequences on the spatial pattern of epidemics. This difference may be linked to current research directions in botanical epidemiology pertaining to disease spread [60], which may be outlined as follows. On the one hand, focal epidemics [93] occur when a homogeneous plant host population (e.g., a field crop) is exposed to inoculum (aerially) dispersed from a source in an isotropic way. In this case, the concept of traveling wave applies: focal expansion results from the radial expansion of a front away from the source with constant velocity, which results from the gradient having a negative exponential shape. On the other hand, the theory of turbulent diffusion suggests that spread should become more efficient as the diseased area expands [60]. The concept of dispersive wave [13, 63] then applies, that is, a wave of disease progress in space with increasing frontal velocity, which has an algebraic (e.g., power) gradient. This concept leads to predicting disease gradients that become shallower as epidemics progress [60], consistent with the early work of Gregory [19].

Environmental factors may strongly affect the parameters of dispersal gradients [19], one of them being the density of the host plant population [5], cultivated either in pure (e.g., [57]), or in mixed or heterogeneous stands [34].

7.5 SOME SIMPLE MODELS IN BOTANICAL EPIDEMIOLOGY

The models discussed above are primarily meant to describe, rather than explain, epidemics as processes. One of the most important contributions to theoretical epidemiology in plant populations was the differential–difference equation of Van der Plank ([71], equation 8.3, p. 100):

$$\frac{dx[t]}{dt} = R_c(x[t-p] - x[t-p-i])(1-x[t]) \text{ (dimension: } [T^{-1}])$$

where x is the proportion of disease (dimension [1]), t is the (current) time ([T]), p is the latency period duration ([T]), i is the infectious period ([T]), and R_c is the basic infection

rate corrected for removals ([T^{-1}]), that is, the amount of new disease generated per (infectious) disease fraction per unit time. This equation introduces two delays, p and i, and thus states that the increase of disease depends on lesions that are not latent any more, and not yet removed from the epidemiological process. Note that the product $R_c i$ is the equivalent of the gross reproduction rate, R_0, commonly used in medical or animal epidemiology [49, 21].

Considering that a host plant stand consists in a number of sites, which may be considered healthy (vacant to infection), latent, infectious, or removed from the epidemiological process, Zadoks (1971) generated a simple simulation model (Fig. 7.4) enabling to integrate numerically Van der Plank's differential–difference equation. Both the two models generated considerable advances in the field. The former enabled theories and concepts to be developed and discussed – until today, see, for example, Segarra et al. [64] – such as the threshold for an epidemic to possibly occur: $iR_c \geq 1$, and relationships among the "epidemiological quintuplet" [91]: x_0 (the amount of initial disease), p (latency period duration), i (infectious period duration), N (the number of effective propagules per lesion per unit time), and E (infection efficiency, the number of new lesion per effective propagule). The latter (Fig. 7.4) in effect is a Suscept–Exposed–Infectious–Removed (Mollison, 1995) model, which allows to explore, for example, the effects of p, i, and R_c = DMFR = NE, where DMFR stands for the "daily multiplication factor" [86, 91], the numerical equivalent of R_c.

Both models also are useful tools to assess management practices to control epidemics [26]. Figure 7.5, for instance, provides a simulated overview of a "typical" polycyclic epidemic, such as a cereal rust, at reasonable values of parameters i, p, and R_c = DMFR. The graphs indicate that variation in p has a very strong bearing on epidemic outcomes, variation in R_c = DMFR also has a very strong effect, but variation in i does not have such strong effects on epidemics. Such results are useful to guide plant protection, in terms of host plant resistance (which may, e.g., increase p or reduce E), in terms of chemical control (protectants will decrease E), or in terms of crop management, which may affect any of the five components of the quintuplet.

Another approach to modeling disease epidemics is that of Brassett and Gilligan [2], which was developed with respect to soilborne diseases. The model combines properties of the monomolecular and logistic models, and can be written as:

$$\frac{dI}{dt} = (k_1 P + k_2 I)(ZN - I) \text{ (dimension : } [N_{\text{host unit}} T^{-1}])$$

where I is the mean number of infected roots per unit area, N is the mean number of plants per unit area, P is the density of inoculum per unit area, and Z is a parameter for the asymptotic proportion of roots that become infected. k_1 and k_2 are parameters representing the intrinsic rates of infection from primary and secondary inoculum, respectively. This type of model has undergone a number of developments, including variation of the strength of the inoculum source and host growth over time, some of which are briefly addressed below.

7.6 REFINEMENT OF MODELS

Only two types of developments are briefly addressed in this section. One deals with botanical epidemics developing over time and space, and the other with stochasticity in botanical epidemiology.

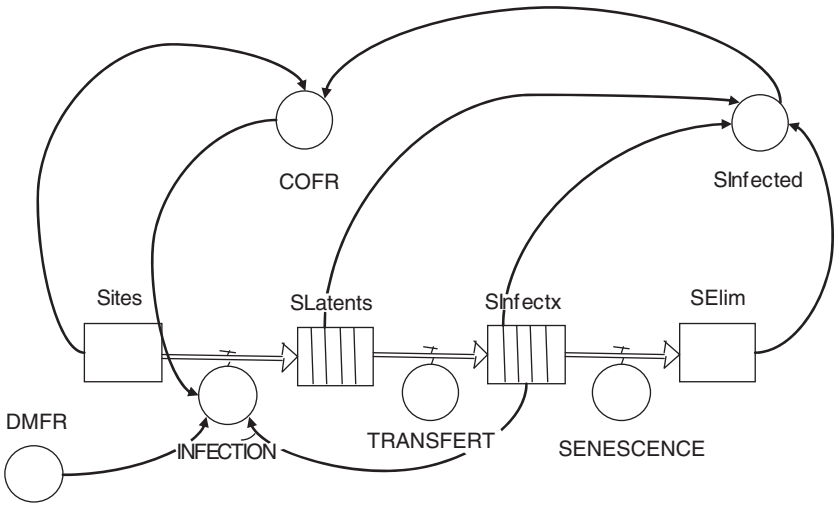

Fig. 7.4. Structure of a simple simulation model for polycyclic epidemics (after [86]). The system often considered is a 1 m^2 of a homogeneous crop. Sites: healthy host units (sites); Slatents: infected, latent sites; Sinfectx: infectious sites; SElim: sites eliminated from the epidemic process (removed); DMFR: daily multiplication factor (number of daughter lesion per lesion per time unit); COFR: correction factor (proportion of healthy sites, relative to the total number of sites of the system); SInfected: accumulated number of victimized sites ; SInfected = Slatents = Sinfectx = SElim.

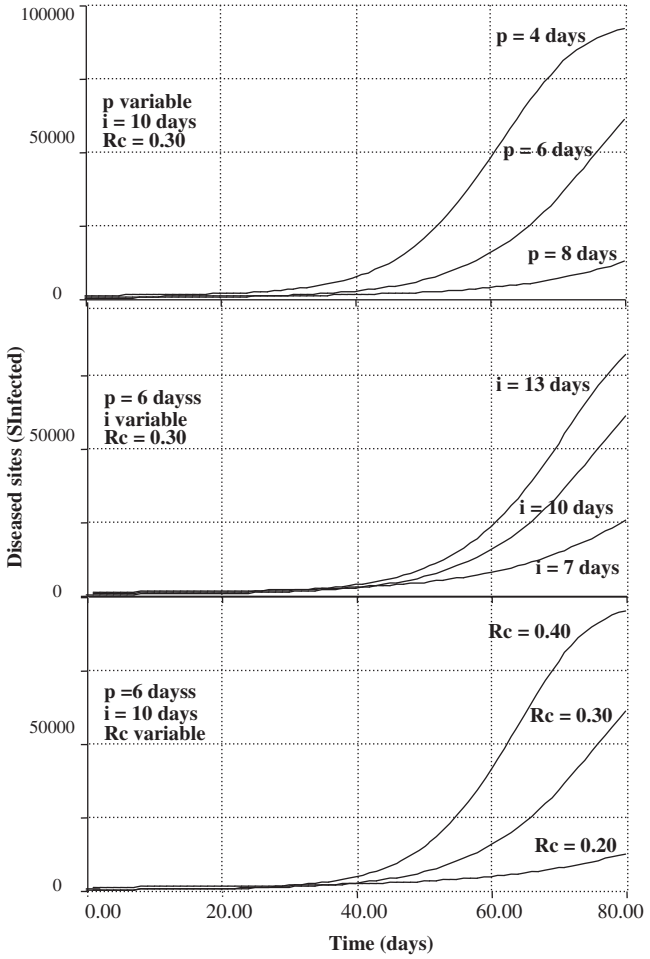

Fig. 7.5. Simulated epidemics, using the simple simulation model for polycyclic epidemics of Figure 7.4. *p*: latency period duration (days); *i*: infectious period (days); R_c = DMFR: daily multiplication factor. Variations in *i*, *p*, and R_c are indicated.

Epidemics in general, and botanical epidemics in particular, depend on dispersive and nondispersive processes, that is, time and space characteristics. Jeger [23] produced a first bridge between strictly temporal models, including Van der Plank's and a series of models describing spatial spread, including Gregory's. This analytical approach is the basis to linking disease variation over both time and space.

Van den Bosch et al. [69] developed a theory of focal expansion based on (1) a time kernel, (2) a contact distribution, and (3) a gross reproduction, where the time kernel describes inoculum production over time and the contact distribution describes inoculum dispersal. Gross reproduction is the total number of victimized (infected) individuals produced by a single infectant (infectious individual) placed in a population consisting of suscepts only (i.e., R_0 [21, 35]). This theory (which involves an exponential dispersal gradient) predicts that a focus of disease expands radially at a rate that asymptotically approaches a constant value.

The spatio-temporal structure of botanical epidemics was studied by Kampmeijer and Zadoks [29] and Zadoks and Kampmeijer [90] using the model EPIMUL. Although the approach of Van den Bosch et al. [69] was a mathematical and theoretical one, the approach in EPIMUL is that of a numerical, mechanistic, and spatially explicit simulation model. This model describes the progress of disease from a source at the center of a lattice of cells (which represent host plant entities, each with a given number of sites), where it can spread and infect new cells. Between-cell disease extensification takes place with a Gaussian dispersal function, and within-cell disease intensification follows the earlier model structure by Zadoks (1971; Fig. 7.4). As indicated by its authors, EPIMUL was not designed to depict reality, but as a tool to explore the behavior of botanical epidemics in time and space, including effects of, for example, diversity or change in the pathogen population. EPIMUL was later used to analyze the epidemiological effects of various strategies for the spatial deployment of genetic diversity in the host population [50]. Another model, involving a spatially explicit model, but with stochastic features, and a contact distribution modeled by a half-Cauchy (i.e., an algebraic, not an exponential) distribution allowed to address similar (initial inoculum, parameters for dispersal, and parameters for disease intensification) and additional (aggregation statistics) issues [77–79].

Spatio-temporal modeling of epidemics may take different shapes, from spatially explicit, to spatially implicit, and from deterministic to probabilistic. This is a field of current and active research.

Within a given host plant stand, botanical epidemics may vary greatly in onset, shape, or speed (see e.g. [43]). Stochasticity is another area of recent developments in botanical epidemiology, where emphasis may concern the inherent variation in disease intensification, the variation of disease onset in a host population during the course of a growing season, and variation in the chance for a host unit becoming infected along a dispersal gradient. Stochasticity may occur at the various levels of integration where an epidemic develops, for example, from spore germination [12], to virus transmission to host plants [14], and to the spatio-temporal development of epidemics (e.g. [42, 65]). Again, this is a field of very active and current research (see, e.g. [18, 62]). A key question is whether stochasticity at one level of integration will lead to divergent behavior of the entire epidemic, seen as a system. This seems to particularly apply when considering the dispersive phase of epidemics, where stochasticity may generate structure at many different scales [62]. In such a context, the gross reproduction parameter (R_0) may need to be larger than 1 in order that an epidemic occurs in a population [21].

7.7 DISEASE MANAGEMENT: A BRIEF REVIEW OF PRINCIPLES

Management of botanical epidemics entails different decisions and actions [91], which may be categorized as tactical (pertaining to a given epidemic in a given growing season),

strategic short term (anticipating a given growing season) or strategic long term (concerning a number of growing seasons, and possibly different scales in agriculture, from an individual farm to a watershed, to a country, and to an entire ecoregion [92]). Typical tactical decisions include, for example, fertilizer management or pesticide applications in a cultivated field, strategic short-term decisions include the choice of a given crop variety to be established in an orchard or a field, while breeding for particular resistance characteristics or a specified ideotype of cultivated plant implies strategic long-term choices.

Botanical epidemiologists continue to address short-term decisions and develop forecasting systems for disease control. Much progress has recently been achieved, bringing compelling arguments for the use of Bayesian decision theory to design real-time disease prediction rules [39]. These rules may be based on (1) disease assessment or measurement of environmental factors which may influence diseases, and (2) predictors, that is, their consequences in terms of epidemic dynamics or losses (using empirical or mechanistic models). The approach derives from processing of diagnosis in medicine, with the additional difficulty that predictions concern epidemics occurring in populations, not disease developing on a particular individual, and therefore has to include an additional level of integration (and of uncertainty). Yuen et al. [84] proposed the use of receiver operating characteristic (ROC) curves as a mean to compare different predictors. The approach centers on the examination of the probability of epidemic occurrence before and after using the predictor [85]. A detailed review of progress in this area is given in Madden [39].

Host plant resistance is a key tool to manage diseases in cultivated ecosystems worldwide. The example of rice, the first global food crop, is a compelling case where plant breeding for host plant resistance has had a major impact in controlling epidemics, in reducing yield losses due to diseases, and improving food security [47]. In Asia, improved crop varieties, including host plant resistance traits, contributed 0.68–0.97% of a total increase in a food crops yield growth rate of 2.11–3.65% per. year during 1960–2000 (the estimated average values for all developing countries for the same period are 0.52–0.86 to a total growth rate of 2.19–3.20% [11]). Deployment of host plant resistance and durability of host plant resistance are major areas of research, which cannot possibly be addressed in any detail here; the interested reader is referred to reviews of the field [27, 54]. Recently, Van den Bosch and Gilligan [70] developed a simple epidemiological model to link population dynamics and population genetics. Their approach enabled them to contrast the conventional definition of resistance durability (the delay from the release of a resistant variety to the take-over of a virulent genotype within the pathogen population), to two new ones, (1) the delay from the release of a resistant variety until appearance and invasion of a new virulent genotype within the pathogen population, and (2) the added value, in terms of agricultural production, generated from the release of a resistant variety (measured as the added number of uninfected host growth). They conclude that the conventional definition may not necessarily be the most useful, and that the two other definitions reflect performances of pathosystems which are, too, strongly dependent on the interplay of population genetics and dynamics.

Policy making and implementation are also examples of long- term decisions for the management of botanical epidemics. Recent results on the rhizomania epidemic of sugar beet in the United Kingdom [66] illustrate this point. A spatially explicit, stochastic simulation model was developed, which considers the polyetic spread of the Beet Necrotic Yellow Vein Virus (BNYVV), which causes rhizomania, a major beet disease. The virus is transmitted by the soilborne myxomycete *Polymyxa betae*. The model considers disease progress at the plant, field, and regional scales, and addresses epidemics over successive years. Spread of the disease at the individual plant level (i.e., root) is caused by zoospores, but spread at any higher scale is primarily due to movement caused by agricultural equipment is, whether within a field, between fields of the same farm or between farms. The model considers the spatial and temporal dynamics of the disease separately. During a cropping season, the disease intensifies (amplifies) at the plant scale, and does not spread; spread occurs between cropping seasons, when no (virus and soilborne vector) amplification occurs. The model designed by Stacey et al. [66] describes the status of all sugar-beet farms and their relationships in the United Kingdom. Outputs of the model were number of infested (i.e., latent, or infected, but asymptomatic), symptomatic, and disease-free fields and farms. The model incorporates both the intensification (amplification) and extensification (dispersal) phases of the disease. The intensification phase, which may last more than a decade before symptoms are detected, was derived from earlier modeling work [68] including parameters for primary and secondary infection at the plant level. Stochasticity was introduced in the description of disease spread. At the field level, the effect of cultivation on the spatial distribution of inoculum was calculated by convoluting [40] the spatial pattern of inoculum with distribution of the probability that an inoculum particle is displaced on a given distance. Spread within and between farms was modeled using Poisson distributions of potentially "infective" journeys of machinery. Several important results were achieved from this work. First, the local containment policy (revoked in 1984), which does not affect the (asymptomatic) front of disease progress, was shown to be ineffective, since by 2050 it would not reduce the spread of the disease. Simulation further indicated that a small reduction (10%) of between-farm cross infection would strongly reduce disease spread. The authors further concluded that any management action must match the scales of epidemics in time and space. In the case of rhizomania, the time scale (delay between infestation and symptom appearance) is 10–15 years, and the spatial scale is the distance on which the disease may be dispersed during this time (about 20 km).

7.8 CONCLUDING REMARKS

Ultimately, the relevance of botanical epidemiology is measured in terms of its contribution to maintaining and sustaining viable ecosystems, whether cultivated or not. The size of this chapter precludes a discussion on the losses caused by plant pathogens, in spite of the fact that it is one key area where concepts of botanical epidemiology meet with concepts pertaining to environmental and agricultural management.

Plant disease epidemics in agroecosystems cause injuries, which may (or may not) lead to crop losses (or damage, i.e., reductions in yield quantity or quality), which, in turn, may translate into losses (reduction in economic return). This cascade of events [89] is the basis of yet another field of investigation of the discipline. Measurement of crop losses caused by plant pathogens is of course a key to measuring the importance of plant pathogens, the need for management of epidemics, and the efficiency of current management [91]. Too often is the measurement of crop losses considered easy and granted; it actually is a field of experimental and modeling investigations of its own. Crop losses may be seen as a yardstick to measure the harmfulness of epidemics, as well as the efficiency of current disease control [37, 38, 67].

Modeling allows simulating the amount of crop loss caused by disease injuries, which vary over time as an epidemic develops over the course of a cropping season; it also allows measuring the effect of varying man-made environment (which defines the attainable performances of a crop) on crop losses. Further, it allows attention to shift away from crop losses and focus instead on yield gains [9]–benefits derived from better crop management, from better strategies involving short-and long-term vision of plant health. Recent modeling work [75], backed with heavy field experimental support, has enabled to analyze crop losses dynamically caused by a range of diseases, insects, and weeds of rice in tropical Asia in a range of production situations, and to project yield gains which novel research could derive. Research policies are also long-term decisions.

Shifts in paradigms have occurred, which nowadays guide research. One of them derives from the realization that elimination of a plant pathogen is neither possible nor desirable in the immense majority of cases [44, 91]; "control" progressively gives way to "management." This notion, which derives from ecological research of the middle of the last century, still holds promises of progress today, even in the current context of biological invasions of plant pathogens, which can only be prevented and managed when they occur (see, e.g. [83]). Another is that one host plant population is seldom exposed to injuries caused by one pathogen only, or more generally, by one harmful agent. Farmers are concerned with many problems other than plant protection; when they do, they often have to consider several of them simultaneously; and ecological principles suggest that empty niches seldom exist [44]: multiple pathosystems are facts, whose shape and composition vary with the man-made environment [58]. The characterization, analysis, and modeling of multiple pathosystems is yet another front for future progress [59]. This will probably require novel ways to address botanical epidemiology, where the concepts of epidemiological guilds and guilds of harmful agents might be useful to address and manage syndromes of production and syndromes of disease.

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CHAPTER 8

Malaria Vaccines

Charles W. Todd,[1] Venkatachalam Udhayakumar,[1] Ananias A. Escalante,[2] and Altaf A. Lal[3]

[1]*Division of Parasitic Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA*
[2]*Arizona State University, Tempe, AZ, USA*
[3]*U.S. Embassy, New Delhi, India*

8.1 INTRODUCTION

Despite a century of campaigns, the worldwide burden of malaria is still staggering. It is one of the most prevalent parasitic infections, and the number of clinical cases is estimated to be over 500 million [36,71]. Mortality in infants and children in Sub-Saharan Africa alone is at least 800,000 annually with the majority of deaths resulting from severe cases of *Plasmodium falciparum*. Additional deaths occur in other malarious regions (Fig. 8.1). It has been estimated that the annual loss of economic growth is 1.3% in countries where malaria is endemic [15], with Africa again bearing the brunt, losing approximately US$12 billion in productivity annually [56]. The current major public health tools to combat malaria are (1) case management, offering diagnosis and treatment of infected persons; (2) infection prevention, using vector control and insecticide-treated bed nets; and (3) disease prevention, for instance, intermittent preventive treatment. When rigorously applied, these methods are effective, but they are constantly threatened by the emergence of resistance to insecticides and antimalarials [84], plus the lack of political will and financial resources to implement and sustain them [6].

These powerful tools will continue to be the foundation of control campaigns; however, it is understood that effective malaria vaccines would provide additional useful and cost-effective ways to help control the morbidity and mortality of this disease complex [23,47].

The development of an effective vaccine has been thwarted, in part, because malaria parasites, of the genus *Plasmodium*, have complex life cycles involving sexual development in the vector mosquitoes and asexual cycles in their vertebrate hosts (see Fig. 8.2). The infective sporozoites pass into the vertebrate hosts while *Anopheles* mosquitoes take a blood meal (Fig. 8.3).

These sporozoites enter the circulation and within 30 min home to the liver where they invade the parenchymal cells. Here they differentiate into hepatic merozoites, a process termed hepatic or exo-erythrocytic schizogony. This stage of infection produces no clinical symptoms. The erythrocytic infection is initiated when hepatic merozoites are released into the blood stream. Within seconds, these merozoites must attach to and invade red blood cells, where they multiply to form multinucleate schizonts (Fig. 8.4).

P. falciparum can invade all red blood cells, whereas *P. vivax* targets reticulocytes. The sequential release of new merozoites, for example, every 44–48 h in *P. falciparum* infection, during erythrocytic schizogony initiates the classical clinical manifestations of cyclic fever, headache, chills, and anorexia. The severity of the clinical outcome depends on the immunity of the host, with children less than 5 years old and pregnant women being the most susceptible in endemic areas. Nonimmune persons including adults are highly susceptible to the development of clinical malaria. After several erythrocytic asexual stage cycles, a subpopulation of parasites develops into gametocytes (Fig. 8.4), which may then be taken up by *Anopheles* mosquitoes with a blood meal. Sexual reproduction in the mosquito and seeding of sporozoites into the salivary glands complete the life cycle. The vertebrate host and its immune system are thus exposed to four stages: sporozoites, liver stages, merozoites, and gametocytes. These stages are generally antigenically distinct, though there is limited overlap. Each of these stages presents opportunities for immune attack, but with limitations and potential consequences.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

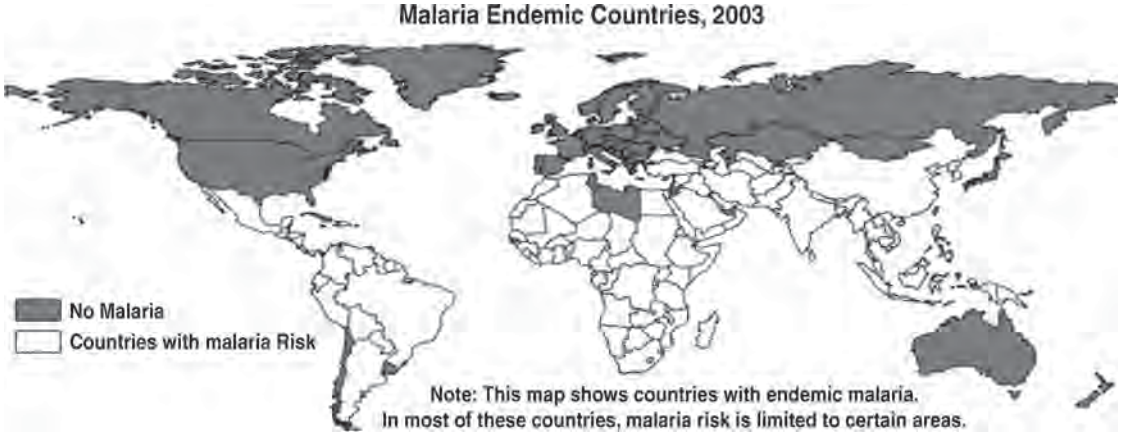

Fig. 8.1. Worldwide distribution of malaria. From www.cdc.gov.

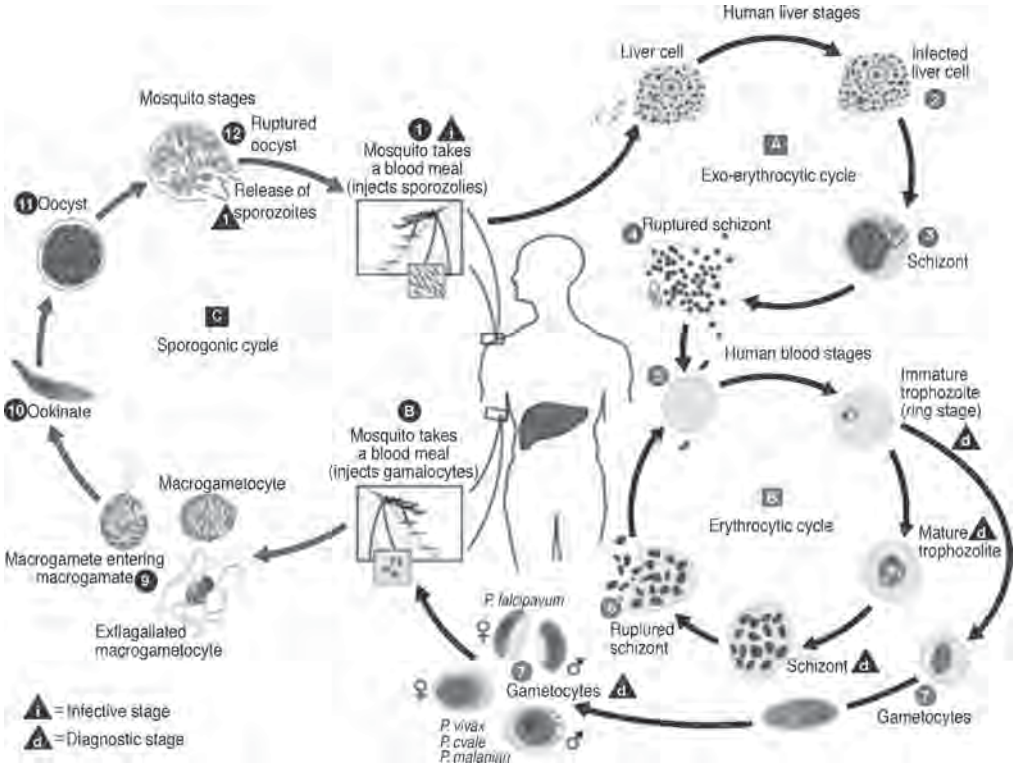

Fig. 8.2. The schema of the life cycle of malaria. The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host❶. Sporozoites infect liver cells❷ and mature into schizonts❸, which rupture and release merozoites❹. (Notice, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes)❼. Blood-stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes❾. The zygotes in turn become motile and elongated (ookinetes)❿ which invade the midgut wall of the mosquito where they develop into oocysts⓫. The oocysts grow, rupture, and release sporozoites⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites❶ into a new human host perpetuates the malaria life cycle. From http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm. See color plates.

Fig. 8.3. A replete *Anopheles* mosquito following a blood meal. From http://www.cdc.gov/malaria/biology/mosquito/frame.htm.

8.2 MALARIA VACCINE CONSIDERATIONS

It is axiomatic that a vaccine functions by inducing immunity to the desired pathogen. Given the complexity of the malaria life cycle, the different stages to which the host is exposed, and the genetic variability of human populations, it is pertinent to ask if people can develop immunity to malaria and thus whether it is possible to develop a successful vaccine. Several lines of evidence suggest that development of a vaccine against malaria is feasible. Persons living in endemic regions, who are repeatedly infected, do develop functional immunity over time [16]. This immunity is generally weak against sporozoites and liver stages, thus it neither prevents reinfection nor eliminates parasites from the blood and sequestered locations. But the acquired immunity does limit the parasitemia and diminishes the toxic effects of parasite products, thereby ameliorating clinical disease and protecting against severe morbidity and mortality [24]. Though it should be remembered that many do not develop immunity, hence the large numbers of deaths of children each year. In addition to acquired immunity, immuno-modulatory activities may protect the human host from potentially deleterious effects of overzealous effector functions directed against the parasite. Therefore, in the setting of natural exposure, the human immune system is capable of controlling malaria sufficiently to diminish its deleterious effect. It is worth reiterating that this functional immunity is primarily directed against decreasing the clinical effects of malaria and reducing the parasite burden; it does not break the cycle of transmission.

In contrast, experimental studies in animals and small-scale human clinical trials have demonstrated that significant immunity can be induced to the sporozoite and liver stages [49]. Immunization with radiation-attenuated sporozoites is capable of stimulating an immunity that absolutely protects certain individuals against a subsequent challenge of fully virulent and viable sporozoites [49], though the immunity wanes in a matter of months. Recent clinical trial results from Mozambique with an antigen derived from the circumsporozoite protein (CSP) also provide early indications in children that a recombinant malaria vaccine can be developed that can potentially reduce the burden of clinical malaria [5]. These observations demonstrate that the human immune system does, under differing circumstances, have the ability to prevent and control infection by malaria, thereby providing justification for vaccine development efforts.

Although it is generally agreed that a malaria vaccine is feasible, there is no consensus in the field concerning the characteristics of an ideal vaccine. Setting aside for now these differences, the malaria vaccines currently under development [7,85] fall into three broad categories, based on the stages of the parasite's life cycle: (1) pre-erythrocytic, (2) asexual blood stages, and (3) transmission blocking, affecting the sexual stages. Pre-erythrocytic vaccines are directed against the invading sporozoite and the intra-hepatic stages, and aim to abrogate the infection before the first wave of merozoites is released into the circulation. Such vaccines may be based on individual antigenic determinants found on sporozoites [5], or on attenuated sporozoites that can stimulate protection but cannot themselves initiate an infection [49]. Animal models and very limited human studies have demonstrated that such vaccines can give complete protection. However, their disadvantage is that should the parasites in just one infected hepatocyte survive, a

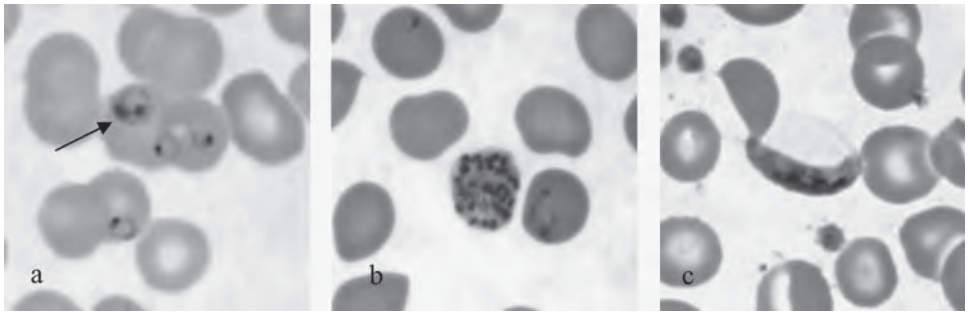

Fig. 8.4. Blood stages of *P. falciparum*. (a) Ring stages, and mature trophozoite (arrowed). (b) Mature schizont. (c) Gametocyte. From http://www.dpd.cdc.gov/dpdx/HTML/Malaria.htm.

situation that could result from either less than 100% protection immediately following immunization, or incremental loss of complete protection with time, a normal erythrocytic infection would be expected to result. Because such a vaccine would have minimal efficacy against blood stages, once breakthrough had been achieved, it would be expected to provide little if any protection against clinical symptoms or pathology [42]. Such pre-erythrocytic vaccines have been proposed for international travelers and military personnel.

Because all of the pathology associated with malaria is a consequence of erythrocytic infection, vaccines against the asexual blood stages have been proposed as a way to decrease the morbidity and mortality of the vast majority of clinical cases. Such vaccines are intended to replicate or accelerate the development of the natural immunity already described that manifests in older children and adults in endemic areas who have survived multiple previous infections [16].

In contrast to the above vaccine types, transmission-blocking vaccines are altruistic in that they do not interrupt or ameliorate infection in the recipient [19]. These vaccines induce antibody responses against antigens on the sexual stages of the parasite. These antibodies do not exert their effect in the human host but pass into the mosquito midgut with a blood meal, where they react with nascent antigens on the gametocyte sexual stages and inhibit ookinete formation and development. The aim of these vaccines is to prevent sexual replication in the mosquito and thereby decrease transmission pressure. Because the gametocyte antigens are not under immune selection, they show limited variability and genetic diversity [27,28].

Although the three main categories of vaccines represent valid approaches, each has its limitations. There is a growing awareness that single antigen vaccines may be insufficient to provide protection, and that vaccines may have to contain both multiple antigens from a given stage and antigens from different stages.

There are essentially three target populations for the proposed malaria vaccines. From the sheer magnitude of the public health problem, the largest and most vulnerable population requiring protection is infants and children under 5 years of age in Sub-Saharan Africa and Southeast Asia [71]. These children bear the brunt of the disease and suffer the greatest mortality. Studies from Western Kenya indicate that anemia, a significant factor in malaria-induced mortality, is prevalent from 6 to 24 months with a peak around 12 months [52,53]. These data demonstrate both the susceptibility of the very young to malaria and that immune interventions to build resistance must be initiated at or very soon after birth in endemic areas. The goal is to develop sufficient immunity in these children by immunization so that they are protected from the worst manifestations of the disease that result in morbidity and mortality.

Pregnant women and their unborn children are both extremely susceptible to the deleterious effects of malaria infection. These women are at increased risk of developing complications compared to their nonpregnant peers [54]. Their infection often presents with a sequestration of parasitized erythrocytes in the maternal placental blood, a condition known as placental malaria, which contributes to both maternal anemia and low infant birthweight. The frequency and severity of placental malaria decrease following successive, malaria-exposed pregnancies, again suggesting that specific immunity can ameliorate the pathology and that vaccine-derived protection would also be of help.

The final groups that would benefit from a malaria vaccine include travelers, including those returning to visit friends and relatives in malarious areas, military personnel, and expatriates. By definition, these groups have the financial resources to protect themselves with antimalarial drugs, and thus ideally, should not be at risk.

8.3 REQUIRED EFFICACY OF MALARIA VACCINES

What efficacy will be required of the different vaccines to achieve licensure and have a significant effect on public health? This consideration will depend on the type of vaccine being developed, the target population, the desired end point, and the epidemiology of malaria in a given locale. Consider a pre-erythrocytic vaccine that provided no immunity to subsequent blood stages being used in an immunologically naive population, for example, travelers. Such a vaccine could induce high titer antibodies to block sporozoite penetration into the liver, cytotoxic T lymphocytes (CTL) to kill infected hepatocytes, or an appropriate combination thereof. It is estimated that a single mosquito bite usually delivers fewer than 100 sporozoites, and that 1 sporozoite-infected liver cell yields 10,000–40,000 merozoites – sufficient to initiate an infection [30]. Modeling studies have indicated that pre-erythrocytic vaccines that reduce the number of infected hepatocytes by 99% would be expected to delay the onset of patent parasitemia by only 4–5 days, and have little subsequent effect on the course of infection [42] and presumably disease. How a 99% reduction of infected hepatocytes translates into true vaccine efficacy (i.e., percentage of persons absolutely protected) has yet to be determined. None the less, these considerations indicate that the requirements for a pre-erythrocytic vaccine may be very stringent. It is also possible that the protection afforded by a pre-erythrocytic vaccine in the face of naturally occurring sporozoite challenge may allow the immune system to develop partial immunity to subsequent blood stages. As mentioned above, the protection afforded by a pre-erythrocytic vaccine may depend on significant CTL activity against infected hepatocytes. Thus, clinical testing of a pre-erythrocytic vaccine in an endemic area would have to monitor liver function very closely to ensure that continued natural sporozoite challenge and consequent CTL activity did not result in liver pathology. Because pre-erythrocytic vaccines are designed to prevent infection, their efficacy will probably be measured by the classical method commonly used for routine vaccines [63].

In contrast to pre-erythrocytic vaccines, blood stage vaccines are currently envisioned to boost the immunity of susceptible individuals where malaria is endemic and to allow them to control parasitemia and significantly ameliorate morbidity and mortality. Currently, interventions to control malaria are evaluated against existing disease burden within a given area. For example, the deployment of insecticide-treated bed nets (ITN) in Africa has reduced the rate of all cause mortality in children less than 5 years of age by approximately 20% [21], ranging from 10% in Burkina Faso [35] to 30% in Kenya [59,72]. Vaccine trials evaluate their efficacy similarly [5]. Although this percentage reduction strategy suffices for the present, it may become prudent to switch to absolute end points as trials progress. For example, in an immunized population of children under 5 years of age, absolute end points could be prevalences of parasitemia $<5\%$, severe anemia $<1\%$, and clinical disease $<5\%$. Similar arguments can be applied to the reduction of placental malaria by immunization. The incidence of placental malaria varies by locale up to a maximum of between 55 and 60% in West Africa for primigravidas and approximately 35% for multigravid women [14,54]. A valid goal for a vaccine would be to reduce the rate of placental malaria to less than 5% in both primigravida and multigravida women in all endemic areas. Because the prevalence and incidence of all these the parameters vary according to the locale, disease epidemiology, and malaria control activities, it would not be appropriate to define universal end points at this time. However, investigators should consider establishing such bench marks appropriate for the specific endemic area when planning clinical trials of malaria vaccines.

8.4 DURATION OF PROTECTION

After Spf66, which had initial promise in South America [57] but failed to achieve significant protection in endemic parts of Africa and Asia [22,60], the RTS,S antigen based on CSP has advanced the furthest in terms of clinical development. Several studies of RTS,S formulated with the powerful adjuvant ASO-2 have demonstrated limited protection in nonimmune individuals [76], semi-immune adults in Gambia [13], and children living in endemic areas of Mozambique [5]. In all studies, the immuno-protective effects of the vaccine are short lived, lasting for a matter of months rather than the years of protection associated with the majority of routinely used vaccines. It is hoped that further clinical trials with this vaccine/adjuvant formulation will either demonstrate increased protection or illuminate its limitations such that improvements will follow.

8.5 FIELD EPIDEMIOLOGY STUDIES

The real challenge is to develop a vaccine for endemic populations that will reduce disease burden and mortality, even though it may not completely eliminate circulating parasites. Testing such a vaccine is also demanding because the manifestations of disease vary in different endemic settings and age groups. In areas with intense annual transmission of malaria such as Western Kenya and parts of Tanzania, severe anemia is the most common serious manifestation and cerebral malaria occurs rarely [52,73]. A community-based study conducted in Western Kenya showed that most of the severe anemia burden was found in children <2 years of old [52–54] and that the overall mortality was higher in infants. In this setting, any malaria vaccine needs to be administered within the first few months of life in order to reduce mortality and morbidity. In areas of less intense transmission such as Gambia, cerebral malaria is the major severe manifestation of malaria and occurs mostly among 2–4-year-old children [32]. Within areas with seasonal malaria transmission, for example, South and Southeast Asia, adults are affected in addition to children and pregnant women.

The complications associated with malaria vary from cerebral malaria and anemia to renal failure and respiratory distress. The underlying causes for the differences in disease manifestations in the different endemic settings are not yet understood. Under these circumstances, it becomes essential that any new vaccine candidate be tested in various sites reflecting different endemic settings and different target groups including infants, children, and adults.

In preparation for such multicenter vaccine trials, community-based cohort studies in different endemic settings need to be conducted to characterize the epidemiology of malaria, immune responses to malaria, parasite characteristics, the genetics of the host population, and the entomological characteristics of the vector population. A multidisciplinary community-based study conducted in the Asembo Bay area of Western Kenya provided valuable information needed for vaccine testing [12]. This study established that most of the mortality and severe anemia episodes occur between 2 and 18 months of life [52,73]. The reduction in the severe anemia episodes and mortality after the first 18 months of life suggests acquisition of clinical immunity. This was further evident from host genetic association studies. It is well established that the prevalence of hemoglobin variants such as the sickle cell trait is high in malaria endemic areas, and this has been attributed to the selective advantage of sickle cell heterozygotes (HbAS) against malaria-associated mortality [3,4]. A large genetic association study conducted in this cohort showed that the HbAS genotype conferred protection against malaria-associated severe anemia episodes only for children between 2 and 16 months of age, corresponding to the age window when the maximum number of anemia episodes occurred in this population [1]. This finding indicated that when clinical immunity develops in the second year of life, the innate protective advantage conferred by the sickle cell trait becomes undetectable. Interestingly, HbAS conferred about 55% protection against overall mortality in this cohort, although it reduced the risk for severe anemia only 60% and high-density *P. falciparum* infections by 25%. Children with HbAS did not show any reduction in the over all prevalence

of parasitemia as compared to children with normal hemoglobin (HbAA). These observations suggest that a vaccine that mimicked the protective effects of the sickle cell trait by reducing the number of high-density infections and anemia episodes early in life could significantly reduce the mortality and morbidity associated with malaria, although it may not alter the overall prevalence of malaria infection.

8.6 SELECTION OF VACCINE EPITOPES

The genetic diversity of *P. falciparum* exhibits a very complex pattern; it is extensive among genes encoding potential vaccine antigens, particularly at those genes encoding surface proteins of the blood stage, whereas there are almost no nucleotide substitutions at housekeeping genes or those antigen genes expressed in the mosquito vector. This high genetic diversity in the gene-encoding antigens of the surface of blood stages has been known for some time [51]. These early findings received further support from a series of studies on selected antigens (reviewed in [25]). In such studies, positive selection by the host immune system was implicated as the potential driving force behind this high, but localized, genetic diversity. Specifically, genes encoding antigenic proteins elicit immune responses; thus, the observed levels of heterozygosity in gene-encoding antigens can be accounted for by the accumulation of mutations that allow the parasite to escape host immune recognition. Although this hypothesis is appealing, little is known about the link between parasite genetic diversity and host immune responses for the vast majority of malaria antigens that have been described.

The most important issue for vaccine developers, especially those who focus on selected antigenic targets (be they recombinant protein or DNA vaccines), is to determine whether or not the vaccine-elicited immune responses will be effective against all genetic variants found in nature. In order to answer this question, two logical steps need to be accomplished for each protein being considered as a vaccine candidate. First, the nature and extent of a protein's genetic variation needs to be assessed so that the factors involved in its generation and maintenance can be explored. This line of research provides information about the extent of diversity an experimental vaccine could encounter while also providing evidence of the importance of natural selection by the host immune response in maintaining the observed polymorphism [25]. If positive natural selection is the most parsimonious explanation for a given polymorphism, then researchers could consider that antigen as relevant to developing natural immunity. In the case of vaccines aiming to elicit sterile immunity, the vaccine antigen should target the parasite in such a way that its induced immunity cannot be eluded by the genetic diversity of the parasite at population level. For such antigens, studying their genetic diversity constitutes baseline information needed to evaluate the level of polymorphism that the vaccine-elicited immune response is expected to face.

The second step is to link such genetic diversity data with immune response outcomes specifically to identify which aspect of the genetic variation really affects immune reactivity and antigen recognition. This will also help to identify how many immunologically different alleleic forms of a given target antigen exist in nature and whether it would be feasible to choose a limited number of these allelic forms of the protein as vaccine targets. Although this issue has been studied for some selected antigens such as the merozoite surface protein-1 (MSP-1), there is a large gap in our understanding of the identity of the most useful vaccine candidate antigens among the thousands of proteins that have been identified from the malaria genome project, and the number of antigenic forms/variants in which the relevant targets exist.

Regarding CTL epitopes, very complex dynamics have been found [2]. For example, in the CSP of *P. falciparum*, critical substitutions can elicit cross immunity among very distant alleles [81]. The situation may become more complicated if we consider that the extensive diversity of these epitopes is just starting to be estimated [26]. It is expected that the dynamic interactions of genetic diversity and elicited immune responses will change from gene to gene. For example, the extensive genetic diversity observed in the gene encoding the apical membrane antigen (AMA-1) appears to be maintained by positive natural selection [27,64,65]. In addition, there is evidence suggesting that natural selection may have geographic specificities [20]. These population genetic analyses have received further support from evidence based on recombinant constructs of this protein. The results derived from these constructs indicate that the genetic diversity in AMA-1 is indeed recognized differently by host immune responses [40]. However, regardless of its extensive genetic diversity, it appears that a limited repertoire of constructs could generate cross immunity among several alleles, making possible the inclusion of AMA-1 in viable vaccine formulations [66]. Thus, careful field and laboratory studies are needed to quantitate this cross immunity in order to include groups of alleles that could potentially be differentially targeted by vaccine constructs.

A cautionary note, however, is that natural selection acts on the available genetic diversity of the malaria parasite within a specific environment. The immune responses in a given human population will be elicited against the circulating parasite epitopes, thus they will be affected by the history of the parasite and human populations, as well as the ecological circumstances in which these two populations interact. Field studies aiming to evaluate cross immunity among groups of alleles need to take into consideration that some vaccine formulations may demonstrate altered effectiveness in different settings.

8.7 THE VACCINE TARGET: EPITOPES OR STRAINS?

The term strain has been widely used wherever a particular group of parasites has been considered as a plausible explanation for a specific clinical manifestation of malaria. Thus, it is

assumed that these particular groups of parasites can be defined in terms of their genetic characteristics. Nevertheless, in terms of vaccine development, strains are defined in terms of a phenotype that is independently transmitted [34]. However, there is still some confusion in the definition of strain-specific immunity. The term "strain-specific immunity" could imply two possibilities: either that linkage among loci encoding antigens is observed and that it is maintained by the host immune response, or that there is no cross immunity among a series of antigenic variants. These specific characteristics allow us to delimit a strain as an independent transmission unit [34]; thus, it has real implications in vaccine design. Following these arguments, because linkage groups can originate by inbreeding if transmission is low [50], before we can claim that a specific group of antigens define a strain, evidence is needed that the observed linkage cannot be explained by the population demography alone. On the contrary, if we are talking about an antigenic variant defined using specific criteria, we need to show that there is limited or no cross immunity among those entities. Nevertheless, we believe that the term "strain-specific immunity" applied to any single antigen generates unnecessary confusion wherever information on cross-reactivity among alleles is limited or absent. Probably a term such as "allele-elicited immunity," specific or not, may be a more appropriate. Thus, epitopes at specific genes with their different alleles are the vaccine targets. This discussion is relevant if we consider vaccines to target a specific group of parasites in order to protect a defined risk group such as pregnant women.

8.8 CYTOADHERENCE AND VARIANT GENES

Cytoadherence of *P. falciparum* parasites to endothelial cells in the blood veins and placenta is a hallmark of this infection, and this characteristic is considered to be an important irulence factor associated with pathogenicity [55]. The most studied antigen associated with this process in *P. falciparum* is erythrocyte membrane protein-1 (PfEMP-1) [55]. PfEMP1 belongs to a family of 250–350-kDa proteins that are targets of protective antibody responses and are encoded by the *var* gene family [8,79]. PfEMP-1-mediated binding to endothelial cells takes place via ubiquitous receptors such as CD36, CSA, and ICAM-1, among others [55]. In the case of placental malaria, it has been proposed that the placenta provides a unique environment that selects for parasite sub-populations expressing CSA-binding [29] and hyaluronic acid-binding PfEMP1 proteins [9]. Specific *var* genes have been implicated as encoding the CSA-binding ligand [17,68], making them suitable targets for a protective vaccine that may reduce the negative consequences of placental malaria. However, the evidence is still not conclusive. Specifically, some receptors are expressed in non-placental infections so they cannot be considered "placenta-specific," whereas others lack expression of the CSA-binding phenotype after being transcribed [69]. Thus, no one factor fulfills all the criteria to be considered functional *in vivo* as a "strain" specific target.

It is important to point out that if adherence in the placenta is a trait under positive selection in the parasite population, it could be a convergent phenotype from different genetic backgrounds. Thus, we may have a common phenotype generated by a series of distinct genetic lineages. The implication of this possibility is that there could be more than one *var* gene encoding the CSA-binding ligand expressed in the placenta. This circumstance could generate "contradictory" linking of one *var* or another depending on the human and parasite population under study. In order to rule out this possibility, comparable protocols need to be established in geographic areas with limited or no gene flow among them. These types of studies will increase our chances to detect *var* genes that are local adaptations. They would also allow screening for the same *var* gene across different host genetic backgrounds providing independent corroborations of their involvement in encoding antigens that preferentially bind CSA. This information may lead researchers to formulate a construct against several var genes despite the existence of some locally defined "strains."

In summary, understanding the population dynamics of the parasite genetic diversity is essential in order to explore the viability of a vaccine targeting a specific clinical disease manifestation such as placental malarial. Populations, and not individuals, are the sampling units to understand these processes, thus comparative studies in different settings are needed.

8.9 LIMITATIONS TO MALARIA VACCINE DEVELOPMENT

There is a consensus that pre-erythrocytic vaccines can be safely evaluated in nonimmune human volunteers. These individuals are immunized then subsequently challenged by exposure to mosquitoes infected with a chloroquine-sensitive strain of malaria [61]. At the first diagnosis of a patent infection, the volunteers are treated. Vaccines that show promise can then be refined and/or scaled up for trials in endemic areas. Similar early phase 1 clinical trials to evaluate blood stage vaccines would necessitate a longer period of parasitemia and are precluded by the severe risk to the volunteers. Although the efficacy of these blood stage vaccines must eventually be determined by controlled clinical trials in endemic areas, much work has been performed to try to evaluate vaccine efficacy in animal models at an earlier stage of development, and to establish serological correlates of immune protection.

In this context, the relevance of nonhuman primate immunization and challenge studies in the development of blood stage vaccines against falciparum malaria is controversial. The most widely used nonhuman primate models employ *Aotus* or *Saimiri* monkeys challenged intravenously with parasitized erythrocytes. Proponents argue that such studies might aid the choice of immunogen, eliminate clinical trials of antigens shown to be non-protective, and provide

a means to validate *in vitro* correlates of protection [77]. Opponents counter that these challenge models are variable and unvalidated, may incorrectly (either positively or negatively) predict vaccine efficacy in humans, are biologically and immunologically inappropriate to evaluate efficacy, and should not be considered to be on the critical path of vaccine development [41]. Resolution of some of these differences will await the testing of an identical vaccine formulation in both a nonhuman primate challenge study and a controlled clinical trial in an endemic area.

Stimulation of the human immune system either by natural infection or with antigenic preparations induces antibody responses that react with plasmodial antigens and may demonstrate antiparasite activity *in vitro*. Assigning a clinical protective role to given antigen–antibody reactivities and defining *in vitro* correlates of protection has proved more problematic. Several potential candidates have been proposed.

It has been known for over 20 years that antibodies to sporozoite surface proteins can inhibit the invasion of the hepatoma cell line HEPG2-A16 by this stage *in vitro* [44]. Studies have demonstrated that both volunteers immunized with certain sporozoite antigens and persons repeatedly exposed to malaria in an endemic area developed antibodies with inhibition of sporozoite invasion (ISI) activity. Although there is a general association of increasing ISI titer and increasing clinical protection with age, a direct correlation has yet to be established. Of concern, one study has suggested that *P. falciparum* sporozoites may invade hepatocytes and HEPG2-A16 cells by different mechanisms, thereby casting doubt on the ISI data collected using the hepatoma cells [43].

The growth inhibition assay (GIA) measures the ability of antibody to inhibit the *in vitro* growth of merozoites in erythrocytes [18,37,58,70]. This inhibition may act to prevent entry of merozoites into erythrocytes, their growth and maturation within the cells, exit from schizont-infected red blood cells, or a combination thereof. Persons infected with malaria or specifically immunized demonstrate reactivity by GIA, though correlations between GIA and antibody titers are variable and the significance of the assay to clinical protection has yet to be determined.

Others have proposed that immunoglobulin and monocytes may play a role through the mechanism of antibody-dependent cellular inhibition (ADCI) [10]. Human cytophilic antibodies of the IgG1 and IgG3 isotypes against merozoite surface antigens induce monocytes to secrete primarily tumor necrosis factor α which can block *in vitro* intra-erythrocytic development at the ring stage [11]. Studies have demonstrated an association between titers of cytophilic human IgG isotypes that are merozoite specific with protection against clinical disease in endemic areas [62,80]. However, the direct correlation of *in vitro* ADCI activity with clinical protection is still lacking. In conclusion, the development and testing of a malaria vaccine is thus frustrated to some extent by the lack of good animal models and validated *in vitro* correlates of protection.

8.10 ADJUVANTS AND ANTIGEN DELIVERY SYSTEMS

As we have detailed earlier, immunity to the liver stages of infection is primarily class I-mediated cellular immunity, whereas protection to sporozoites, blood stages, and gametocytes is more dependent on antibody responses (reviewed in [78]). It is very likely that specialized adjuvants and delivery systems will be required to generate the spectrum and magnitude of immunity required for efficacy of a malaria vaccine. When formulated with the appropriate antigens, the classical alum adjuvants (various forms of aluminum hydroxide, aluminum phosphate, and aluminum hydroxyphosphate) have saved millions of lives and stimulate high titer antibody responses (reviewed in [33]). These adjuvants may suffice for malaria vaccines dependent solely on antibody for their efficacy. Adjuvants that favor stimulation of cellular responses tend to be more complex and many are still undergoing development. Saponins, such as Quil A and QS-21 induce more mixed responses, with a significant cellular component, and favor the expression of IgG subclass antibodies that are most efficient at opsonization [45,46]. QS-21 has been formulated with mono phosphoryl lipid A (MPL) in an oil-in-water emulsion to give the proprietary adjuvant ASO-2 [82]. The CSP-derived antigen RTS,S formulated with ASO-2 adjuvant has shown preliminary efficacy against malaria [5]. This formulation induces both potent antibody responses and interferon-γ – a surrogate for cellular immunity [82]. The perceived need for high titer responses has led to the use of Montanide ISA-720, a squalene based water-in-oil emulsion vaccine, with several malaria antigens [31,47,83]. Water-in-oil emulsions have classically been associated with high titer responses, but their increased reactogenicity raises safety concerns, especially because infants and pregnant women are major target populations.

Nanoparticle calcium phosphate (CaP) adjuvants have the potential to target antigens selectively to class I or class II pathways, depending on the formulation. The best class I-associated CTL responses are induced by surface-loading antigen onto CaP nanoparticles [39]. In contrast, the best class II antibody responses occur with slightly larger CaP nanoparticles where the antigen is both core loaded and surface loaded [38]. The potential thus exists to selectively target antigen(s) to both processing pathways by combining the formulations.

8.11 MULTISTAGE, MULTIEPITOPE MALARIA VACCINE CANDIDATE ANTIGENS

Following the rationale that the functional immunity developed by persons living in endemic areas is directed against different stage-specific antigenic determinants, we previously developed a multicomponent, multistage recombinant *P. falciparum* vaccine candidate antigen [74,75]. Such antigens are intended to induce immunity to all stages of malaria occurring

in humans, and thus increase the chances of preventing infection and/or disease. Another advantage is the ability to include combinations of epitopes that will work together to minimize the inherent genetic restrictions of human immune responses, while maximizing human reactivity to genetically diverse *P. falciparum* populations.

8.12 DESCRIPTION OF FALVAC-1 ANTIGEN

FALVAC-1 is a candidate malarial vaccine expressed by a synthetic gene coding 12 B cell, 6 T-helper cell, and 3 CTL epitopes from sporozoite, liver, asexual, and gametocyte stages of *P. falciparum*, and a T-helper epitope from tetanus toxin. The protein is 41 kDa in weight and hydrophilic, and has a theoretic isoelectric point (pI) of 6. The composition of the multivalent, multistage FALVAC-1 antigen is presented in Table 8.1.

TABLE 8.1. The Components and Amino Acid Sequences of FALVAC-1

Epitope[a]	ID[b]	Amino acid sequence[c]
Start		M
Melittin		GIGAVLKVLTTGLPALISWIKRKRQQ
His-tag		HHHHHH
CSP	B	KPKHKKLKQPGDGNP
TRAP	B	WSPCSVTCG
CSP	Th/Tc	KPKDELDYENDIEKKICKMEKCS
CSP	Th	DIEKKICKMEKCSSVFNVVNS
MSP-1	B	NSGCFRHLDEREECKCLL
MSP-1	B	EDSGSNGKKITCECTKPDS
LSA-1	Tc	KPIVQYDNF
CSP	B	NANPNANPNANP
AMA-1	B	DGNCEDIPHVNEFSAIDL
AMA-1	B	GNAEKYDKMDEPQHYGKS
RAP-1	B	LTPLEELY
LSA-1	Tc	KPNDKSLY
P2-T	Th	QYIKAANSKFIGITEL
MSP-2	B	SNTFINNA
MSP-2	B	GQHGHMGH
EBA-175	B	NEREDERTLTKEYEDIVLK
AMA-1	Tp	EFTYMINFGRGQNYWEHPYQKS
AMA-1	Tp	DQPKQYEQHLTDYEKIKEG
Pfg27	B	KPLDKFGNIYDYHYEH
RAP-1	Tp	SSPSSTKSSPSNVKSAS
RAP-1	Tp	LATRLMKKFKAEIRDFF
MSP-1	Tp	GISYYEKVLAKYKDDLE

[a]The origin of the epitope. CSP, circumsporozoite protein; TRAP, thrombospondin related protein; MSP-1, merozoite surface protein-1; LSA-1, liver stage antigen-1; AMA-1, apical membrane antigen-1; RAP-1, rhoptry-associated protein-1; P2-T, tetanus toxoid universal helper; EBA-175, erythrocyte-binding antigen-175; MSP-2, merozoite surface protein-2; Pfg27, gametocyte 27-kDa antigen.

[b]The immune reactivities of the epitopes: B cell (B), T-helper (Th), cytotoxic T cell (Tc), and T proliferative (Tp).

[c]The sequence of each epitope is indicated. The sequence of the entire molecule runs continuously down the column.

Using recombinant FALVAC-1 antigen produced in the baculovirus expression system by Protein Sciences Corporation, immunogenicity studies were conducted in mice, rabbits, and monkeys with different adjuvants [67,74,75]. This vaccine antigen was found to be highly immunogenic in all these three species of animals. The magnitude of immune response also varied in different adjuvant groups. All antisera recognized FALVAC-1 antigen and individual epitopes at various levels. It was found to be immunogenic in the different genetic backgrounds of both inbred and outbred strains of mice, suggesting a low degree of restriction of immune responsiveness for antibody responses. We also investigated the affinity of the vaccine-elicited antibodies in a Biacore assay. Antigen–antibody binding showed multiple kinetic components distinguishable into fast (450–500 s) and slow (800–1400 s) components, indicating that both high- and low-affinity antibodies were produced. Immune responses stimulated by water-in-oil formulations containing the copolymer CRL-1005 demonstrated higher levels of high affinity (i.e., rapidly binding) antibodies. All immune sera showed reactivity with *P. falciparum* sporozoites, asexual blood-stage parasites, and gametocytes. We also conducted ultrastructural localization studies of antibody reactivities to sporozoite, liver, and asexual blood stages of *P. falciparum* using immuno-electron microscopy. Numerous gold particles were obtained on the surface and within the cytoplasm of sporozoites. Antibody recognized parasitophorous vacuole membranes and the cytoplasm of exo-erythrocytic stage parasites. In infected erythrocytes, gold particles were found in rhoptries, on the surface of merozoites and in the cytoplasm of trophozoites. These results demonstrate that antibodies induced to the epitopes in this recombinant molecule can react with those epitopes that are naturally expressed in the different stages of the parasite's life cycle.

The antiparasitic effects of vaccine-elicited antibody responses in rabbits were tested by the ISI and ADCI assays. Purified anti-FALVAC-1 antibodies inhibited sporozoite invasion. Results of the ADCI experiments showed that the vaccine-elicited antibodies had significant effects on *in vitro* growth of blood-stage parasites in the presence of monocytes. No growth inhibitory effects in the absence of monocytes were observed. In conclusion, our initial studies in rabbits showed that FALVAC-1 vaccine antigen is highly immunogenic and that vaccine-induced antibodies recognized native parasite antigens and have anti-parasitic effects.

Proliferative lymphocyte responses were detected in outbred and all three strains of inbred mice but at variable levels. IFN-γ response was also found in both inbred and outbred strains of mice. These results showed that FALVAC-1 is able to elicit cellular responses in outbred mice, and individual variation in cellular response suggests that the response may be partially genetically restricted. Furthermore, QS-21 was found to be the most potent adjuvant in eliciting overall cellular response. It should be noted, however, that studies

with FALVAC constructs in *Aotus* monkeys have yet to demonstrate significant protection to challenge with blood-stage parasites. The appropriateness of nonhuman primate studies to evaluate candidate malaria vaccine antigens has already been discussed. Thus, the final resolution of the utility of the FALVAC antigens as malaria vaccines may require human clinical trials under conditions of natural exposure to mosquito-derived sporozoites.

In summary, our studies for the first time demonstrate that a multiepitope, multivalent recombinant protein can elicit strong immune responses in different animal species, recognize sporozoites and blood-stage parasites, and show protection in *in vitro* assays. These studies have provided evidence for the proof of concept that several epitopes from malaria parasites can be combined and produced as a recombinant antigen for use as potential vaccines.

8.13 DESIGNING ARTIFICIAL RECOMBINANT ANTIGENS

There are many logistical issues with respect to designing a vaccine using the string of epitopes or domains of antigens. First, there are no simple rules to guide the order of the epitopes to produce a vaccine candidate antigen that would induce ideal immune responses and protective immunity. Second, the optimal size of such vaccine candidates for inducing the desired immune responses is not clear. Third, it is becoming evident that introducing spacer amino acids in between selected epitopes may help to increase antigen processing and presentation. Although presently the design of such an antigen is subject to more intuitive rather than rational approaches, several points can be considered at the design stage. One of the most important considerations is the stability of the antigen. Even the most potent vaccine candidate antigen has to be a stable molecule for it to be used in the field. Using computer modeling techniques, one can develop algorithms that would predict a stable structure for a recombinant protein that can be made from a given set of epitopes. In this context, the stability of the molecule may be an important determinant in shaping the ultimate sequence of the epitopes in an artificial vaccine. Another important consideration from safety standpoint is to avoid amino acid sequences that are similar to any host protein because they have the potential to generate autoimmune reactivity. Therefore, any given combination of epitopes also needs to be verified for any such overlap with host proteins. When FALVAC-1 was considered for potential clinical development, the stability of the molecule became a concern for further development. Therefore, we redesigned that molecule using computer algorithms and introducing new spacer sequences. This modified vaccine candidate is referred to as FALVAC-1A, and this molecule has demonstrated significantly improved stability. Immunization studies in rabbits, monkeys, and mice also showed it to be highly immunogenic.

The lessons learned during the development of the multiepitope vaccine antigens and advances in computer modeling of polypeptide structures have been applied to produce more stable molecules that retain their antigenicity. Such studies improve the prospects of the design and testing of multiepitope vaccines that may be able to provide broad spectrum immunity against malaria.

ABBREVIATIONS

ADCI:	Antibody-dependent cellular inhibition
AMA-1:	Apical membrane antigen-1
CD36:	The collagen type I receptor and thrombospondin receptor; a fatty acid translocase involved in long-chain fatty acid transport, fatty acid metabolism and insulin function
CaP:	Calcium phosphate
CSA:	Chondroitin sulfate A
CSP:	Circumsporozoite protein
CTL:	Cytotoxic T lymphocyte
EBA-175:	Erythrocyte binding antigen-175
GIA:	Growth Inhibition Assay
ICAM-1:	Intercellular adhesion molecule 1
IgG:	Immunoglobulin G
ISI:	Inhibition of sporozoite invasion
ITN:	Insecticide-treated bed nets
LSA-1:	Liver stage antigen-1
MPL:	Mono phosphoryl lipid A
MSP-1:	Merozoite surface protein-1
MSP-2:	Merozoite surface protein-2
PfEMP1:	*P. falciparum* erythrocyte membrane protein 1
Pfg27:	Gametocyte 27-kDa antigen
P2-T:	Tetanus toxoid universal helper
RAP-1:	Rhoptry-associated protein-1
TRAP:	Thrombospondin-related protein

GLOSSARY

Adjuvant: A compound or formulation administered with an *antigen* that can augment the immune response to that *antigen*.

Allelle: One member of two genes at a specific locus on a chromosome.

Anemia: A pathological deficiency of hemoglobin in the blood.

Antibody: An immunoglobulin secreted into the blood or lymph in response to an *antigen*, and capable of specific combination with that *antigen*.

Antigen: A substance that elicits a specific immune response when administered to a person or animal.

Attenuated sporozoites: *Sporozoites* that have been treated with ionizing radiation such that they can still invade the liver, but cannot complete intra-hepatic development to *merozoites*. As such, they are a potential pre-erythrocytic vaccine.

ASO-2: A proprietary *adjuvant* consisting of mono phosphoryl lipid A and the *saponin QS-21* in an oil-in-water emulsion.

B cells, *B lymphocytes*: Bone marrow-derived lymphocytes that, when stimulated by *antigen*, differentiate into plasma cells secreting *antibody*.

Cerebral: *Part* of the brain.

Class I-mediated immunity: Immunity mediated by *T lymphocytes* that directly recognize pathogen-infected host cells by engaging the major histocompatibility complex class I molecules.

Class II-mediated immunity: Immunity mediated by helper *T lymphocytes* that typically provide helper function for *B lymphocytes* destined for *antibody* production by engaging the major histocompatibility complex class II molecules.

Cytoadherence: Attachment to cells.

Cytophilic: Having an affinity for cells.

Cytotoxic T lymphocytes: Differentiated *T cells* that can recognize and kill infected target cells displaying a specific foreign antigen derived from, for example, an infecting virus or malaria parasite.

Epitope: A defined region of an *antigen* that specifically stimulates *antibody* induction and combines with that antibody, or that binds to *T lymphocyte* receptors.

Erythrocyte: A red blood cell.

Gametocytes: The stage of malaria found in the blood which when taken up by an *Anopheles* mosquito initiate the cycle of sexual reproduction by producing microgametes and macrogametes.

Hepatocyte: A liver parenchymal cell.

Heterozygote: A person or animal with dissimilar *alleles* at a given chromosomal locus.

Hydrophilic: Having an affinity for water.

Isoelectric point: The pH at which a protein in solution has an overall zero surface charge.

Isotype: One of the *IgG* subclasses, defined by the properties of their constant regions.

Merozoite: A stage of malaria found in the blood that invades other *erythrocytes*.

Monocyte: A large, circulating, amoeboid white blood cell, capable of engulfing and destroying bacteria, protozoan parasites, and dead tissue cells.

Multigravid: More than one pregnancy.

Ookinete: The motile stage of malaria formed by the union of a microgamete and a macrogamete that then penetrates the gut wall of *Anopheles* mosquitoes to form an oocyst.

Opsonization: Facilitation of the phagocytosis of microorganisms by the binding of *antibodies*.

Phenotype: The expression of a specific trait, such as an antigenic variant, based on genetic and environmental influences.

Polymorphism: The occurrence of different forms, for example, antigenic variants in organisms of the same species, independent of sexual variations.

Primigravid: First pregnancy.

QS-21: A proprietary *saponin adjuvant*.

Recombinant (with respect to proteins or antigens): Produced by genetic engineering methods.

Rhoptry: Tubular organelle at the apical complex of a *merozoite*.

RTS,S: A pre-*erythrocytic recombinant antigen* composed of the carboxyl-terminal half of the *P. falciparum* circumsporozoite protein, including part of the central repeat sequence "R" and major *T cell epitopes* "T," which is fused to the entire surface antigen "S" of the hepatitis B virus, and co-expressed in yeast as a particle with non-fused S antigen.

Saponins: A group of plant glucosides with detergent-like properties, from which such *adjuvants* as Quil A and the proprietary *adjuvant QS-21* are derived.

Schizogony: The asexual production of schizonts by multiple mitotic divisions.

Sickle cell disease: An inherited disorder of abnormal hemoglobin resulting in distorted (sickle shaped) *erythrocytes* that in *heterozygotes* provides a measure of resistance to malaria.

SPf66: A malaria vaccine *antigen* consisting of a synthetic protein with amino acid sequences derived from pre-*erythrocytic* and asexual blood-stage proteins of *P. falciparum*.

Sporozoite: The stage of malaria injected into a person or animal by the bite of an *Anopheles* mosquito that subsequently invades the liver. See also *attenuated sporozoites*.

T cells, *T lymphocytes*: Lymphocytes that mature in the thymus and can recognize peptide antigens through their cell-surface receptors.

Trophozoite: One the intra-*erythrocytic* forms of malaria parasites.

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CHAPTER 9

The SARS Case Study: An Alarm Clock?

Gabriel Turinici[1] and Antoine Danchin[2]

[1]*CEREMADE, Université de Paris-Dauphine, Place du Maréchal de Lattre de Tassigny, 75775 Paris Cedex 16, France*

[2]*Génétique des Génomes Bactériens, URA2171 CNRS, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France*

9.1 SARS: DEFINITION AND CLINICAL ASPECTS

All animals suffer from infectious diseases stemming from the development of microorganisms belonging to four major categories: parasites, fungi, bacteria, and viruses. In general, it seems that important changes in the ecological niche occupied by an animal result in the development of new diseases [44]. Although most diseases appeared to have coevolved with the branching of animals during evolution – this is illustrated by tuberculosis, caused by *Mycobacterium tuberculosis*, which probably existed well before domestication of cattle [7], some seem to have emerged suddenly. The "Black Death" is an illustrative example. Although it seems difficult to identify its exact origin, phylogenetic analysis has shown that it probably evolved from the much less dangerous *Yersinia pseudotuberculosis* complex, with progressive loss of genes, from the ancient *Y. pestis* subspecies antiqua, to the subspecies medievalis, and the modern subspecies orientalis [1,46]. However, because the reservoir of the agent is large, the disease could only come under control because it was mostly spread through vectors (fleas). In contrast, smallpox (which appeared very long ago, as witnessed by scars present on pharaoh Ramses' V mummy [29]) could be eradicated because there existed an efficient prevention after the experiments of Jenner, and the widespread use of vaccination with a viral strain that had only limited (but real) side effects. Or, rather, we could think it was eradicated [18] until we decided, unwisely, to sequence the genome of the virus. This publicly available data can allow reconstruction of infectious viral particles [17]. In general, we share diseases with warm-blooded animals, and this explains why the practice of butchery seems to be at the origin of unexpected diseases, such as AIDS, now suspected to have arisen from the common use of "bush-meat" [2] in association, of course, with worldwide changes in human behavior. In this broadly outlined context, an outbreak of "atypical pneumonia" affected the Guangdong province of China in the autumn of 2002, and subsequently resulted in a worldwide outbreak under the common denomination of severe acute respiratory syndrome (SARS) [31].

After some controversy (see, e.g., elements of the discussion here [12,23,47]), SARS was identified as a viral respiratory illness in humans associated to a coronavirus [20,34], previously unknown, finally called SARS-associated coronavirus (SARS-CoV). To the best of our knowledge, the illness spread from November 2002 from the Guangdong province to the rest of China and to the world, with a puzzling contagion behavior. Initial rumors about a dangerous atypical pneumonia in the Guangdong region spread through phone SMS from December 2002. One of its noteworthy features was that health workers were often affected. Early in February 2003, the French Consulate in Guangzhou (Canton) closed a high school for fear of contagion. A few days later, the outbreak reached the Hong Kong SAR (China Special Administrative region of Hong Kong). The following months witnessed the extension of the disease to many countries in North and South America, Asia, and Europe, reaching the status of a worldwide epidemic. One of the difficulties of identifying the disease was to find its specific clinical description (pneumonia are frequent in winter time [32]) and to tell it apart from an episode of H5N1 avian flu that affected patients treated in Hong Kong exactly at the same time [41]. Identification of the SARS Co-V followed by the confirmation of the importance of the epidemic. The initial findings were corroborated by other techniques such as immunostaining, indirect immunofluorescence antibody (IFA) assays, and reverse transcriptase-polymerase chain reaction (RT-PCR)

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with sequencing of a segment of the polymerase gene. Other WHO laboratories found similar results.

The etiologic agent responsible for SARS was identified as a novel coronavirus in late March 2003 by researchers in laboratories from Hong Kong, Germany, and many other countries [20,34,43,47], and its genome was rapidly sequenced by a Canadian team [39]. The new coronavirus was isolated in cells from patients with suspected SARS, having direct or indirect links to the SARS outbreak in Hong Kong or Guangdong Province, China, and was identified initially as a coronavirus by electron microscopy (EM) (Fig. 9.1). Despite an unfortunate spirit of intense competition, an initiative from the WHO, the "World Health Organization Multicentre Collaborative Network for Severe Acute Respiratory Syndrome (SARS) Diagnosis," allowed its members to work together from different research sites through videoconferences and audioconferences and secured Internet web sites. The spread of the epidemic was unconventional, in that different places in the world where contamination occurred had quite different patterns of contagion, morbidity, and mortality. In addition, one observed that children were spared by the adverse effects of the disease. An initial event, traced back to a hotel in Hong Kong, appeared to be the source of most foci in the world, including destinations very far away from one another such as Singapore, Hanoi (Vietnam), and Toronto (Canada) (see Fig. 9.2). The disease spread back from the Guangdong region to Mainland China, Beijing in particular, but not to densely populated regions such as the Shanghai region, despite its intense contacts with Guangdong. Mortality was also very different in different places, with the highest death toll in Hong Kong. This remarkable variability may be due to overreaction of some medical doctors who proposed aggressive treatments in the absence of deep knowledge about the cause of the disease. It could also be due to lack of proper identification of SARS patients, because their status was initially established mostly using clinical and epidemiological criteria only

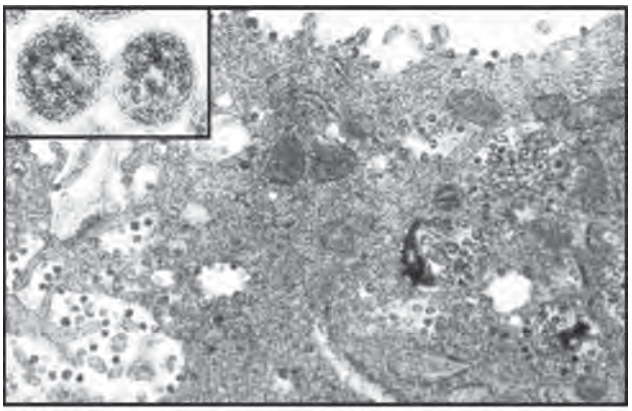


Fig. 9.1. EM of the SARS Co-V (reprinted from [11]).

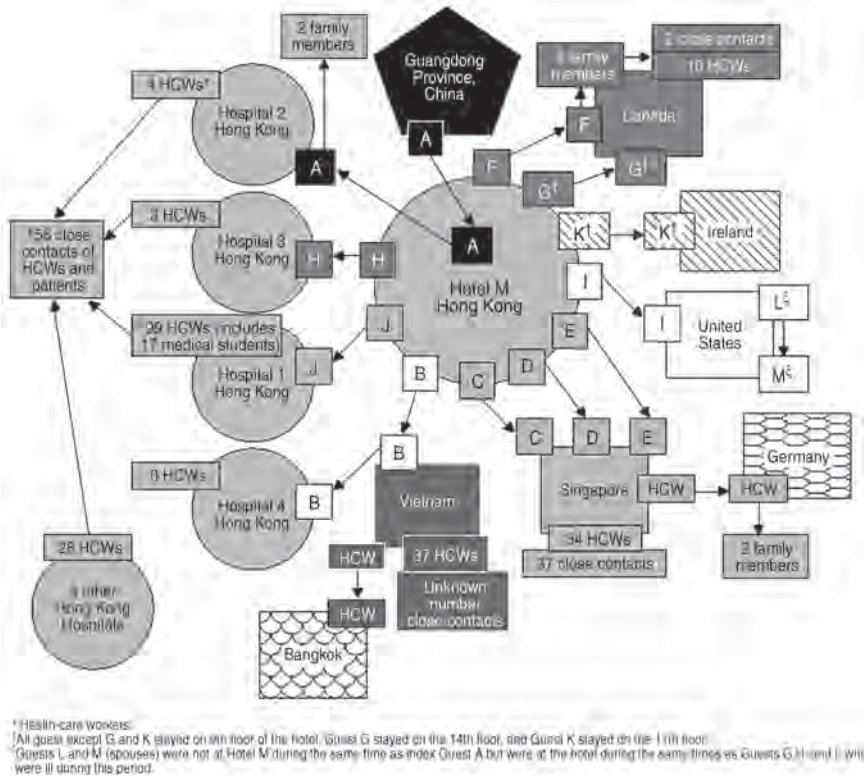


Fig. 9.2. Chain of transmission (reprinted from [11]).

(see [34], supplementary Appendix 1). Retrospective studies indicated that use of the antiviral ribavirin did not improve the condition of patients [37]. A thorough retrospective analysis of the use of steroids is still missing, but anecdotal evidence suggests that continuous supply of steroid might not be optimal [42,59].

Epidemic investigation traced the epidemic evolution back from the patient A. Arrows indicate infection spread either by generating new secondary cases from an index patient or due to the index patient travel. Many of the initially infected cases generated a large number of secondary infections.

Finally, a retrospective study of the sociopolitical context of the time, using information spread through the mass media in addition to that present in fast publication tracks of major scientific journals should be undertaken. It would provide extremely important lessons on the way the world is responding to a highly contagious emerging disease.

The global SARS outbreak of 2003 was finally contained by July 5, 2003, when the WHO reported that the last human chain of transmission of SARS had been broken. Apart from several laboratory accidents causing the re-apparition of the disease (in Singapore and Taiwan in 2003 and in Beijing in 2004), a new SARS episode started late in December 2003 in Guangzhou. Because of the previous experience on SARS, the evolution of the cases, the virus (in molecular terms), and the treatment [62] could be followed in some details. The discovery of the presence of the virus in civet cats enticed some researchers to quickly identify those animals as the source and possible reservoir of the virus [61]. However, several other animals from live animals markets were also found to have been contaminated, and analyses of possible contamination of civet cats in the wild were negative [64]. A retrospective molecular epidemiological study developed by the Guangzhou Center for Disease Control and Prevention, the SARS Consortium of the Minister of Agriculture of the Chinese Central Government and their colleagues showed that the virus genome evolved as fast in civet cats as it did in humans. This was particularly important in that, although civet cats might have contributed to disease transmission, the study strongly suggested that the reservoir is not particularly that animal species [57]. Civet cats, apparently, were contaminated at the same time as humans.

Because they are predators, the obvious inference is that the reservoir is probably a rodent or, with less probability, another small mammal or even a bird. In this respect, the discovery of a highly related virus in Chinese horseshoe bats in Guangdong [35] may be particularly revealing, as bats are not related to rodents (despite their name as "flying mice" or "flying rats" in several languages) but related to Primates, in the superorder Archonta. However, the way the virus might have come into contact with humans is not clear. Bats are used for traditional medicine, and the local population has the habit to eat all kinds of animals. However, among many possible scenarios, they might have been victims of a predator, such as civets (bats are frequently the victims of domestic cats), which might then have passed the virus onto humans. Analysis of the virus genome is consistent with a fast evolution and frequent host shifting [52,65,66] (see Fig. 9.3). This biological background has to be borne in mind when considering the epidemic spread. History of previous coronaviruses epidemics is of particular interest in this context. In the years 1984–1985, an outbreak of respiratory coronaviruses, causing mostly an inapparent infection, spread through the swine population in Europe and then in the United States [36]. Most interestingly, the tropism of the virus had shifted from the gut to the respiratory tract. The change in tropism was the result of a few deletions in the virus genome [50]. Both the parent and the mutant forms later on circulated in porcine herds [33]. This shows that coronaviruses are prone to change in tropism, with concomitant change in virulence. Although truly new emergent diseases can, and will, occur, it is very important to place humans at their place in the phylogeny of animals. In particular, lessons from diseases appearing in domestic animals should be included in the surveillance of human emerging diseases, as they may indicate routes followed by pathogens to spread to animal populations, humans included [58].

We close this introductory part with a brief classification of viruses; we refer the reader to other chapters of this volume for details on phylogeny (contribution by J.R. Stevens and T.A. Richards), unicellular and pluricellular parasites (chapter by F. Thomas et al.), or bacteria classification (contribution by R. Piarroux and D. Bompangue). The metaphor of the "genetic program" is so apt to describe life that, at least at a conceptual level, cells can be described as computers making computers. Within this frame of thought, three "operating systems" would define the three major empires of living organisms, the Archaea, the Bacteria, and the Eukarya. To each of those are associated pieces of program, viruses, that have reproduction as a main goal, in a more or less selfish way. This is why, returning the metaphor, computer sciences speak about "viruses" to describe such pieces of software that propagate through computer networks.

In living organisms, viruses cannot simply be pieces of software, they need to be made of some material, and that material needs to be protected by an outer shell (which can have several names: capsid, envelope, etc.) and designed to recognize a particular target cell. The minimal genetic program of a virus consists of a replication system, and one or usually several proteins involved in the capsid formation (including appendages such as tails, spikes, etc). Because viruses need to interfere with their host cells, their genome often codes for many proteins interfering with the metabolism of the host, diverting it to permit virus development. In some cases, they even code for metabolic enzymes (such as thymidine kinase in herpes viruses [6]) or enzymes or factors of the translation machinery (such as translation initiation factors, aminoacyl-tRNA synthetases or tRNAs [13]). However, they neither code for the core of the translation machinery nor of the core

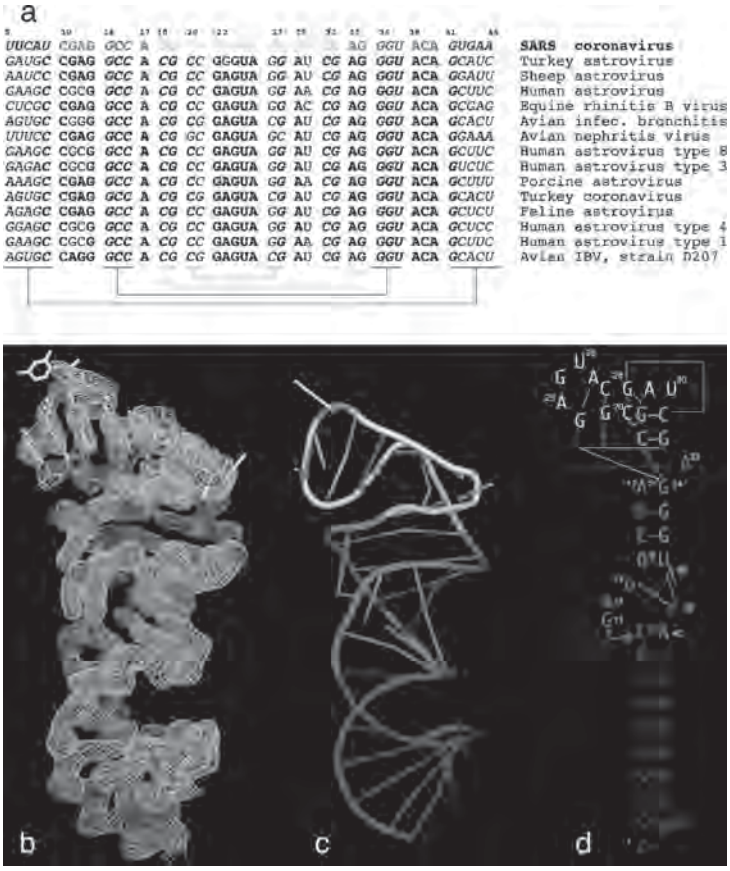


Fig. 9.3. (reprinted from [54]). The primary, secondary, and tertiary structures of the SARS s2m RNA. (A) Phylogenetic comparisons of RNA sequences from various viruses. The SARS RNA sequence is color coded to match the color scheme used throughout. Conserved sequences are highlighted as bold letters, and co-varying sequences involved in conventional RNA helical base pairing are indicated in italics. Sequence complements are indicated using color-coded brackets. (B) Experimental electron density map contour that allowed unambiguous tracing of the RNA molecule. (C) A corresponding ribbon diagram highlighting the unusual fold. (D) Schematic representation of the RNA secondary structure with tertiary structural interactions indicated as long-range contacts. See color plates.

metabolism, making them necessarily parasites, and, as such, not endowed with life. Some viruses can integrate the host genome as proviruses, and stay there until some signal triggers their development. This latter feature is particularly important, as it means that those viruses can lay dormant for a long time (and even throughout generations) and be suddenly reactivated, creating havoc. They can become defective, and unable to reproduce, but this ability can be restored by recombination with an active virus, creating a variety of new variants, or simply by functional complementation. Hence, a remnant of a provirus in a genome can never be considered as completely innocuous. Associated with these properties, the following general classification has been proposed:

- The double-stranded DNA viruses (e.g., Adenoviridae, Herpesviridae).
- The single-stranded DNA viruses (e.g., Parvoviridae).
- The DNA and RNA reverse transcribing viruses (e.g., Hepadnaviridae, Retroviridae).
- The double-stranded RNA viruses (e.g., Reoviridae).
- The negative single-stranded RNA viruses (e.g., Bornaviridae, Filoviridae, Paramyxoviridae).
- The positive single-stranded RNA viruses (e.g., Coronaviridae, Picornaviridae).

In the latter category in particular, viruses can have a segmented genome. This is the case of viruses important for health such as the influenza viruses and the hantaviruses.

Other related agents, such as satellites or viroids are not described here. A universal system for classifying viruses, and a unified taxonomy, has been established by the International Committee on Taxonomy of Viruses (ICTV) since 1966 [30]. The system makes use of a series of ranked taxons, in a classical cladistic way:

- Order (-virales) being the highest currently recognized.
- Family (-viridae).
- Subfamily (-virinae).
- Genus (-virus).
- Species (e.g., SARS coronavirus).

Although the spread of the 2003 SARS outbreak was of less important magnitude than other worldwide epidemics, it has attracted attention due to its special characteristics that suggested the need for tailored approaches both in theoretical modeling and in clinical practice. Interest for the disease was also triggered by the high mortality of the infected patients [19] and its apparent resistance to standard approaches, resulting in worldwide negative economic consequences. However, the overall reaction of the health care and researchers communities in the world was remarkably positive in that the virus was identified only a few weeks after the first cases were discovered. Furthermore, the outbreak was put under control in a few months time. Whether this is due to proper reaction of sanitary authorities or the particular features of the virus and disease remains yet to be explored, both with theoretical epidemiological models and molecular epidemiology studies.

The symptoms of the SARS, that created its name, correspond to a highly virulent disease. Beside patients with a limited contagion pattern, some patients were super spreaders who caused many secondary infections. However, should one consider every SARS-infected individual as a super spreader, the disease would soon have been out of control; fortunately, this is not what happened, as many people seemed to be shielded from infection by some unknown circumstances. Common sense suggests that stricter hygiene conditions would necessarily contribute positively to widespread protection with epidemic propagation being blocked at places with strict sanitary policy. In contrast, if we analyze the reality of the disease propagation, we must note that medical personnel, air travelers, and airport personnel were among the most affected by the disease, whereas other, less specific social environments seemed to go unaffected.[1] The phenomenon is reminiscent of the "herd immunity" concept central to the theoretical simulations of epidemic spread (we will come back to this, with further details, on the mathematical modeling in the next section). At its origin, this concept was used to explain why, during the course of an epidemic, some individuals do not develop the disease even if they are not immunized against it. In such a description, the epidemic results from a balance between the speed of propagation and the responsiveness of the quarantine and other health policy measures, and the number of individuals that are not infected by the disease is determined by these parameters. For the SARS 2002–2003 episode, the propagation of the disease suggested some sort of pre-existing protection, but its cause and explanation still remain to be found. Nevertheless, it was observed that, contrary to expectation, places with lower hygiene seemed protected against SARS, whereas places with more strict sanitary conditions were mostly affected. Furthermore, and this still requires an analysis, children and younger adults did not have signs of the disease.

Before going into specific analysis of the virulence and infectivity of the SARS-CoV, let us point out another circumstance that affects the long-term evolution of the disease. As is the case in the paradigm of ecological biosystems, an equilibrium often tends to govern the relationship of the virus with its host [24–26,51,60]: if the virus is too virulent, then it will prevent further transmission by the host (e.g., because the host dies or is rapidly quarantined). If, in contrast, the virus is less virulent, it will not be able to reproduce itself efficiently. The equilibrium can be either static, where the levels of virus and the host stabilize to some constant values or dynamic when those values evolve in time in (periodic) cycles, as in the simplest predator–prey Lotka–Volterra model. This model describes interactions between two species in an ecosystem, a predator and a prey, and prescribes the equations that model the evolution of the populations of prey and predator [63]. The introduction of an additional species in an ecosystem and its effects have received some attention [16,24]. Note, however, that convergence to a stable or periodic steady state does not appear immediately but needs time to setup; in the meantime, the evolution of the epidemic can be supposed to happen at constant virulence and interaction parameters. For the SARS, it could be argued that a dynamics fitting the standard model was established starting with the second epidemic (2003–2004) because the virus was less virulent; the equilibrium pattern was not apparent in the first 2002–2003 epidemic, so that the standard model cannot explain its dynamics. Other factors have thus to be taken into account.

Let us come back now to the factors that may explain the differences in infectivity under various hygienic conditions. Studies show that the genetic characteristics of the virus have varied [15] during the course of its spreading. This evolution, triggered by the lack of adaptation of the virus to its new human host [57], must have had an impact on its infectivity. It may also have influenced its fitness, as the virus emerged in a localized region and did not yet propagate through different hosts and conditions. From this point of view, the epidemic can be seen as a (averaging) process in which the virus optimizes its characteristics to maximize its chances of

[1] An outbreak of Marburg hemorrhagic fever, caused by a filovirus, affected Angola during the first semester of 2005. Interestingly, as in the case of SARS, the hospital where patients were treated became a source of major contamination. "On 9 Apr 2005, an international medical charity battling the hemorrhagic fever that so far has killed 181 Angolans has urged the government to close the regional hospital here, at the center of the outbreak, saying the medical center itself is a source of the deadly infection. 'Médecins sans Frontières' (MSF), the global relief organization that runs a n isolation ward at the hospital for victims of the deadly Marburg hemorrhagic fever, told Angolan officials on Friday [8 Apr 2005] that the hospital should be closed if the rapidly spreading epidemic was to be contained. Two other hospitals within 60 miles of Uige may also have to be shut down (according to M. de Astellarnau, the organization's emergency coordinator in Uige, the provincial capital where the outbreak was first reported)."

survival in the whole population. However, in the absence of accurate data on the evolution of these precise genetic dynamics, a first approach would be to consider its simplest form where different viruses can be introduced and affect the entire population.

Building on historical data on a set of coronavirus-mediated epidemics that affected pigs in the 1983–1985 [36], Ng et al. [40] introduced the assumption that two simultaneous epidemics interacted. The hypothesis of the *double epidemic model for SARS* that they introduced was based on the high mutation and recombination rates of coronaviruses [28], and on the observation that tissue tropism can change by simple mutations [50] (see Fig. 9.4 for situating the SARS-CoV among other known pathogens).

A Bayesian inference phylogeny of the nucleocapsid protein of coronaviruses, compared with the phylogeny of their hosts (lines drawn between the two phylogenies indicate the host status of each coronavirus), suggests that the SARS-CoV could have resulted both from host-switching and tissue tropism change. This analysis is also consistent with a significant role of recombination [66]. At the time of this analysis, the sequence of Chinese horseshoe bats coronavirus was not known, but we can infer that it would fit extremely well in the picture, as bats are highly related to primates, whereas their coronavirus is highly similar to SARS-CoV [35].

Interaction between both epidemics required involvement of a considerable proportion of the population; accordingly, the first epidemic was supposed to be extremely contagious. As this is often the case with the oro-fecal route, such an epidemic could be propagated by contaminated food, contaminated water, or sewage. It could be caused by some coronavirus, call it virus A. Among its manifestations, examples of visible symptoms would be gastroenteritis (this was consistent with the observed medical data during the winter of 2002–2003 Guangdong and in Hong Kong where many people had diarrhea for about 1 day, but certainly not substantiated by explicit data). This hypothesis is to be related to the above considerations on the optimal balance between the virulence (the facility with which the virus propagates to generate new cases) and aggressiveness with respect to the host (the consequences of the disease in terms of host's health). To ensure its existence even beyond host's death or recovery, an "older," genetically stable virus, would likely display more of the first and less of the second. This perfectly fits with our description. Indeed, it is expected that a virus would rather be moderately pathogenic while retaining the possibility to spread very easily and not the reverse. An additional virus, call it virus B, is responsible for the SARS epidemic. One possible cause for the origin of the virus B is a genetic operator (recombination or more probably mutation [28]) applied to virus A [3,4,22]. Because the virus B is not yet in a stabilized form, its propagation and characteristics

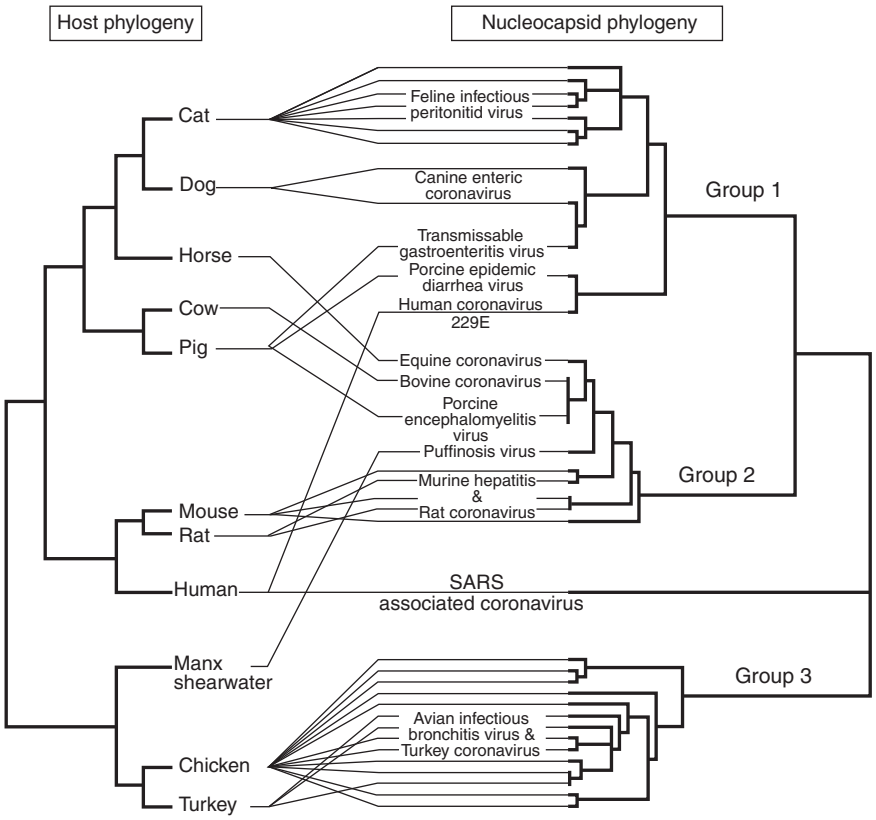
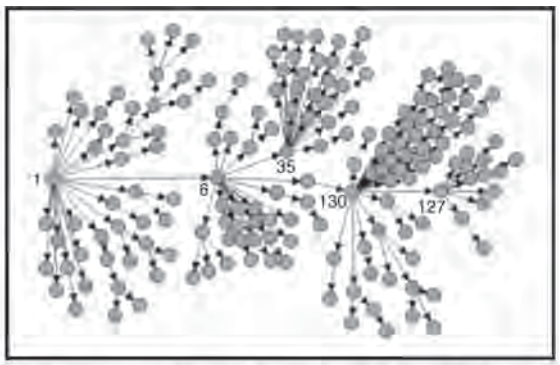


Fig. 9.4. Phylogeny of the nucleocapsid protein of corona-viruses compared with the phylogeny of their hosts (reprinted from [52], copyright (2003), with permission from Elsevier).

are likely to be very different from those of the virus A: virulence should be high to compensate for the small quantity initially produced, but aggressiveness can also be important because it is not yet correlated, through the host dynamics, with virulence. A distinct situation would appear when the viruses have different origin but generate cross-reacting immune responses of the host. In both situations, the epidemics would spread in parallel; because of the common genetic structure or similar host response, it can be expected that the first epidemic would protect against the SARS (so that naïve regions not protected by the virus A can get large SARS outbreaks). These assumptions, which generate a specific spreading pattern of this double epidemic hypothesis, are to be compared to puzzling distribution of the disease evolution in Asia and, for example, the pronounced difference in the status of Shanghai, Beijing, and the mainland. The hypothesis is also to be related with more local characteristics of the spread, as witnessed by the existence of some very infectious individuals but the absence of a worldwide mass epidemic, simultaneously with high infectiveness of health care workers. The environments with less strict hygienic conditions are more likely to be infected with virus A and therefore protected from SARS, whereas in hospitals, the virus A will not gain ground and thus the population will be naïve, thus sensitive to virus B. We will come back to the mathematical description of the model and the fit with the observed results. In a different form, a number of authors speak about "unsuspected SARS patients" [31] that were identified early in the epidemic in Singapore [9] and later in Taiwan [10]. These cases have either atypical symptoms or could not be immediately related to known cases of SARS [38]. These patients may have turned out into reservoirs and affected the latter propagation of SARS. During the course of the epidemic and in the following months, several studies [38,53,56] addressed the structure of the epidemic spread and computed the model parameters that would explain the data. These analyses estimate first the basic reproductive number R_0 that is defined as the expected number of secondary infections generated by an average infectious case in an entirely susceptible population. We propose in Figure 9.5 below a graphical illustration (see also [14]).



* Patient 1 represents Case 1;Patient 6, Case 2; Patient 35; Patient 130, Case 4; and Patient 127, Case 5. Excludes 22 cases with either no or poorly defined direct contacts or who were cases translocated to Singapore and the seven contacts of one of these cases.
Reference: Bogatti SP. Netdraw 1.0 Network Visualization Software. Harvard, Massachusetts: Analytic Technologies, 2002.

Fig. 9.5. Chains of transmission (reprinted from [14]).

When $R_0 > 1$, the epidemic will spread, or otherwise terminate. The parameter was found to be initially above 1 (and thus the disease has the potential to spread to a majority of population), and it then evolved to less than 1 during the course of the epidemic. This change is argued to follow the implementation of the public health policies. Other basic measures that have been investigated are the time from onset of infection to hospital admission or from onset to appearance of clinical symptoms.

9.2 MATHEMATICAL MODELS FOR EPIDEMIC SPREAD PROPAGATION

The mathematical description and modeling of the epidemic spread has been tailored to explain the important characteristics of the disease evolution and its impact on the population. Several descriptions are currently in use depending on the precise practical circumstances, and it is beyond the scope of this chapter to exhaustively address them all. It is nevertheless important to give a brief overview of the methods available to the researcher and on the phenomena that it is possible to transcribe nowadays into mathematical models.

The model that has historically been among the first to capture an important epidemiological phenomenon is the so-called "SIR" model. Its assumptions are fairly simple: the total population is constant in time and can be divided into three classes:

- The "susceptibles," denoted by S, that is, the people that are naïve with regard to the disease (neither had it nor are immune to it).
- The "infectives" denoted by I, those that have been contaminated with the disease. It is supposed that on entering this class, the members can instantly propagate the disease. Also, at the individual level, the disease is considered to begin displaying symptoms and doing its inner work without further delay. We will see later that these assumptions can be relaxed in the "SEIR" model.
- The "Removed" class, denoted by R, contains the people that have had the disease and are either dead or in quarantine, that is, they have been set apart from the entire population and cannot transmit the disease any longer.

Any individual is completely described by specifying the S, I, or R class to which she/he belongs: no further individual differentiation is considered. Every individual in a given class is interchangeable with any other in the same class. The additional ingredient necessary to implement this model is to prescribe how the transition is operated among classes. The overall scheme is the following: from "S" class to "I" class and then to the "R" class: $S \rightarrow I \rightarrow R$. The transition between two classes is governed by the following rules:

- In a given small time interval $[t, t + dt]$, the transition from "S" to "I" is proportional to the number of S and I encounters (as measured by the product SI) and to the time span "dt." In its simplest mathematical transcription, each of the classes S, I, and R is a time-varying number and its evolution is represented by a simple ordinary equation: $dS/dt = -rSI$. If, on the contrary, the evolution is considered stochastically, then the associated stochastic event moves one individual between the classes S and I: $(S, I) \rightarrow (S - 1, I + 1)$. The probability for such an event to appear has an exponential distribution of parameter rI for each member of the class S.
- Besides the incoming dynamics originating from S, the individuals in class I can be affected by their migration to class R. This is supposed to be proportional to the number of individuals in class I, resulting in the evolution equation $dI/dt = -bI$, or, at the stochastic level, the event $I \rightarrow I - 1$ is an exponentially distributed random event with parameter b for each individual of the class I. This results in the dynamics of R class to be $dR/dt = bI$.

The deterministic variant of the model described above results in the following general form for the evolution of the classes S, I, and R: class S decreases until its final value Sf; class I increases and then decreases; and class R monotonically increases to its final value Rf. The fundamental strength of the SIR model is to capture the so-called "herd immunity": although there is nothing hardcoded into the model to prevent the total initial naïve population $S(0)$ to be infected, it turns out that the final Rf value is less than its maximal possible value, or in other words, S_f is strictly positive. The epidemic extinguishes not because it is short of susceptible individuals, but because, at some point during the epidemic, the infected individuals are removed faster than they are infected. This can be seen from the equation of the classes

$$dS/dt = -rSI$$

$$dI/dt = (rS - b)I$$

$$dR/dt = bI$$

where dI/dt decreases (and thus epidemic is extinguishing) as soon as $rS(t) < b$. We recover the basic reproductive number $R_0 = rS(0)/b$, which can be interpreted as the number of secondary infections produced by one primary infection in a whole susceptible population; at a later time "t," the effective reproductive number R_t $rS(t)/b$ can also be introduced. We obtain the fundamental criterion to decide of the state of an epidemic: $R_0 > 1$ means propagation, $R_0 < 1$ means epidemic extinction.

The deterministic model is justified when the epidemic is of large size. In this regime, it can also be regarded as the limit of the stochastic model, which can also be used for smaller sized classes. The meanings of these two models are slightly different: in the deterministic setting, the uncertainties have been averaged out and only the mean dynamics is retained. As such, the simulation is expected to mimic empirically observed figures. On the contrary, in the stochastic setting, each simulation is a possible scenario but all are equally possible. It is crucial to realize that no individual stochastic realization but their averaged trajectory is to be taken as predictor for future evolution of the epidemic. Furthermore, in addition to this average, the stochastic model can also provide the estimate of the deviation from the mean dynamics.

Building on this first SIR epidemic model, it is possible to refine it by including additional classes. An often-used extension is to consider the class of exposed individuals to be placed between S and I. This model applies to diseases with incubation period such as SARS. The flow of individuals between consecutive classes is $S \rightarrow E \rightarrow I \rightarrow R$, and the corresponding equations are as follows

$$dS/dt = -rSI$$

$$dE/dt = rSI - bE$$

$$dI/dt = bE - aI$$

$$dR/dt = aI$$

The interpretation of the new parameters is as follows: $1/b$ is the mean time for an individual to stay in the E class, that is, the mean (incubation) time from infection to onset of symptoms (that is supposed simultaneous with infectiousness); $1/a$ is the mean time from onset to hospital admission (or quarantine, or death). These parameters have been estimated for SARS [19], yielding a mean incubation period of 6.4 days (95% CI 5.2–7.7), whereas the mean time from onset of clinical symptoms to admission to hospital varied between 3 and 5 days, with longer times earlier in the epidemic. The same study also provided an estimate mortality rate between 6.8% and 13.2% for patients younger than 60 years and 43.3% and 55.0% for patients aged 60 years or older.

To identify the parameters, the model is fitted to the observed number of hospital admitted cases. These cases are reported per day which, with the above notations, means the values $R(n + 1) - R(n)$ for $n = 1, \ldots$ In mathematical terms, fitting the evolution given by the theoretical model to the observed data is an "inverse problem," which can be recast as an optimization process. This problem may have multiple solutions, and therefore care is to be taken when analyzing the resulting parameters. This is particularly the case for intricate models, which, because of their complexity, will fit virtually any data set (and in particular the actual one) with possibly several solutions for each. Then, the existence of a fit does not by itself necessarily prove that the model is realistic. By contrast, a model that associates a unique solution (possibly with error bars) to a given data set is expected to carry some similarity to the actual dynamics.

The models discussed above stress the importance of the rapid identification and isolation of infected individuals as a mean to control a general epidemic.

Beyond these general theoretical considerations, these models have been used to predict the future course of the epidemics and to asses the impact of the measures taken to contain it. For the SARS 2002–2003 epidemic [38] (see also [21]), data from Singapore and Hong Kong allowed estimation of the reproductive number R by supposing an exponential growth in the number of cases and provided hints of its time evolution. It was found that the epidemic had potential for infecting a large part of the population if not controlled and thus justified the necessity for enforcing stringent health policies. However, due to the presence of super spreaders (individuals who generate many more infections than the average), the estimations of the reproductive numbers still carried large error bars (wide confidence intervals). To further document the efficiency of the health policies, among which quarantine, the same authors introduced subsequently a compartmentalized model similar in spirit to SEIR but with additional classes differentiated over quarantine conditions.

Continuing this analysis, a different approach was taken in [53] that also computed the reproductive number (found as around 2.7 at the beginning of the epidemic if super spreaders are excluded). The paper subsequently evaluated the impact of the public health interventions and argued that the decrease in the reproductive number R was mainly driven by reduction in population contact rates and improved hospital infection control.

Further refined, epidemic specific, health policies can also be assessed if additional spread characteristics are included in the model; these specificities result from collaboration with on-field specialists to allow validation of the hypotheses and make critical use of the highest quality epidemiological data. It is essential for such studies to be made possible during the course of the epidemic. Thus, the data has to be readily available not only to clinicians but also to the scientific community as a whole, in an effort to secure a rapid and timely improvement of the public containment policies.

For the SARS epidemic, additional models are required to explain the long-term persistence of the virus [21] and its spatial transmission differentiation as well as the super-spreader events.

9.3 THE DOUBLE EPIDEMIC MODEL

As an illustration of a model that takes into account the possible existence of a differentiation among the population exposed to the SARS epidemic, we will briefly present below the double epidemic model introduced in [40]. This approach considers that a protective factor exists that can prevent SARS infection even after exposure to the virus. This protective factor is expressed as acquired immunity due to a previous infection with a different coronavirus (or another immunologically cross-reacting virus) that manifests (mildly), for example, as a gastroenteritis that can easily go unnoticed. We will follow the notation of the previous section and design by A the initial mild virus and by B the SARS-CoV. If both viruses have a common structure, it may be possible that individuals infected with the virus A acquire immunity with respect to SARS-CoV. These individuals may either be asymptomatic but propagate the SARS or even completely prevent further SARS propagation. It is the latter hypothesis that we consider here, which results in the decomposition of the total population into subclasses described in the flow chart of Figure 9.6. The class S contains initially the whole population, the $S \longrightarrow E \longrightarrow I \longrightarrow R$ branch models the SARS, whereas the competing branch $S \longrightarrow I_P \longrightarrow R_P$ models the protective epidemic of virus A. This results in the driving equations:

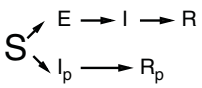


Fig. 9.6. Flow chart of the individuals through mutually disjoint classes in the double epidemic hypothesis.

$$\mathrm{d}S/\mathrm{d}t = -rS(t)I(t) - r_P S(t)I_P(t)$$

$$\mathrm{d}E/\mathrm{d}t = rS(t)I(t) - bE(t)$$

$$\mathrm{d}I/\mathrm{d}t = bE(t) - aI(t)$$

$$\mathrm{d}R/\mathrm{d}t = aI(t)$$

$$\mathrm{d}I_P(t)/\mathrm{d}t = r_P S(t)I_P(t) - a_P I_P(t)$$

$$\mathrm{d}R_P(t)/\mathrm{d}t = a_P I_P(t)$$

Depending on the initial conditions set on the above dynamical model, the protective epidemic can act through two qualitatively distinct scenarios:

- As a "static" protection where initially a large part of the population is immunized (and belongs thus to the class R_P).
- Or as "dynamic" protection where the virus A spreads simultaneously with the SARS: people first infected with A will be protected from SARS, whereas others will remain naïve to it.

This model fitted the data in Hong Kong, Beijing, and Inner Mongolia, and it was seen that both types of protections gave realistic results, with the "dynamic" alternative replicating better the qualitative form of the curves. In all cases, the main epidemiological parameters (basic reproductive numbers, incubation/latent periods, time from onset to hospital admission) were searched for and fit was obtained in ranges compatible with the previous studies.

The fit itself is realized through the optimization of a cost functional F(.) i.e. a function that associates to a given set of parameters the distance between the simulated data (corresponding to the set of parameters) and the actual observed

data (in our case the curve R(t)). This information is fed into an optimization algorithm that finds the set of parameters which minimizes the value of $F(\cdot)$. Because in general there is no analytic formula to operate this inverse mapping, numerical optimization algorithms are used. Standard algorithms include gradient steps [48] or Monte Carlo approaches [55]; additional examples of search procedures use genetic-like algorithms [27] or modified simplex algorithms [5]. It should be noted that often the underlying mathematical optimization problem is difficult, with many suboptimal local optima (imperfect solutions that cannot be improved with local moves), and it is difficult to ensure that convergence to the best possible set of parameters is achieved.

9.4 CONCLUSION

Although the SARS 2003 outbreak was small when compared to other epidemics, its evolution attracted much interest from the public and was followed on a daily basis by people worldwide. During its evolution, the fundamental question was whether the implemented health policy measures successfully worked toward containing the disease. Its special characteristics, namely the presence of super spreaders and the high number of lethal cases among health care workers suggested that much of its evolution was inconsistent and not yet understood at the epidemiological level, whether in its clinical or modeling facets. Combined with the observation of propagation through air travel, such a belief negatively oriented the perception of the potential of the disease to affect a large part of the global population.

Under such circumstances, scientific analyses are crucial, from the very beginning of an epidemic, to provide efficient directions to set up appropriate control measures. As society evolves, the theoretical tools available from classical epidemiological studies have to be adapted to the new socioeconomical conditions. For instance, the costs of containment measures such as quarantine, especially those incurred by the airlines companies, and the losses due to the absence of expected tourism-generated income in affected areas are not negligible and have to be taken into account when designing a control strategy. These socioeconomical parameters may even have a negative impact on data availability, as some local authorities and even governments may be tempted to underreport or declare the epidemic contained too early. To analyze such phenomena, situated at the interface of health policies, economics, and politics, data should be released to scientists at all possible levels. Furthermore, although theoretical methods are likely to exist nowadays to tackle these subjects, meaningful insights and data are often only directed to specialists of a precise discipline (e.g., economic data to economists, health care data to epidemiologists, etc.), preventing a global approach to the situation. As far as possible, an effort is likely necessary from all sides to fill this information gap.

The same comments apply also to the clinical studies. Although national, specialized research institutes remain a necessity, cooperation with foreign teams has proven to be instrumental to rapid advances, for example, to the sequencing of the virus genome, just to cite one. The need for appropriate international collaboration in the field of influenza research, at a time when many fear a new pandemic triggered by the H5N1 virus, is absolutely essential [8]. A complementary point of view would also emphasize that the structural configuration of the clinical research should always allow not only intra-disciplinary mutual enterprises but also inter-disciplinary research with monitoring alternative strategies being a mean to accelerate implementation of meaningful advances. Indeed, epidemiology has a singular standpoint in the field of science because it not only has to deliver verified scientific truths but also deliver them fast enough to be operational for the control of the ongoing epidemic. Splitting the effort into component tasks and listening to all relevant ideas are certainly key to future advances. Of course, once the epidemic is over, the background work that prepares adequate responses to the next epidemic is also crucial. The SARS 2003 epidemic showed that the scientific community can find the tools to react quickly to the demanding tasks raised by an emerging disease. These tools are still perfectible, however, and have to be adapted to address the inevitable future challenges posed by similar epidemics, particularly, flu. It has long been established that flu is a normal, usually innocuous, disease of Anatidae (ducks, geese, and the like). It can spread to other birds, and when this happens, the disease, as expected when the host changes, becomes more virulent initially and then attenuated (this is the normal course of any infectious disease, and this property has been used for the creation of many vaccines [45]). In some cases, the disease can jump to mammals, usually pigs (they are bred, in China, together with ducks in the backyard of farms) and then to humans (remember the Chinese character for "family": a pig under a roof, symbol of the normal happy situation of a farmer). When this happens, we have one of those dangerous episodes witnessed from time to time, and most often coming from Asia, for that very socioeconomical reason. Now, for the present H5N1 strain story, we know (and this is the same for the H7N7 strain [49]) that there was first contamination of poultry (not only Anatidae but also several kinds of fowls: this is why it was advocated in Hong Kong, as early as in 2001, to monitor scavenging birds such as *Milvus migrans*, as sentries for the propagation of the virus), then direct contamination of humans. Because the virus is not adapted to humans, it causes a very extreme reaction, ending, unfortunately, in death. But for that very reason, the virus does not (yet) multiply in humans in such a way that it would cause human-to-human contamination. It is when the virus will have mutated to a less lethal form, it is likely that it will start spreading from humans to humans, and trigger the pandemic many people are afraid of. Whether a "double epidemic" scenario may happen in this case remains to be seen.

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CHAPTER 10

Recombination and Its Role in the Evolution of Pathogenic Microbes

Philip Awadalla,[1] Xin-zhuan Su,[2] and Kate McGee[1]

[1]*Department of Genetics, North Carolina State University, Raleigh, NC, USA*

[2]*Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

10.1 INTRODUCTION

Many microbes have sexual stages that can lead to sexual recombination in their genome. For example, 13% of the protein-coding portion of the largely asexual reproducing species, *Eschericia coli*, is thought to be the product of lateral transfers from other organisms. Even the smaller genomes of *Helicobacter pylori* and *Haemophilus influenzae* have a substantial portion of their genomes, 6.2% and 4.5%, respectively, that are considered "foreign" in origin. These transfer events are likely ancient in origin, but intraspecific analyses have demonstrated that recombination events happen frequently within many viral, bacterial, and parasitic protozoan species as well [20,29,39]. Pathogenic microbes are often subject to extreme selection pressures, have short generation times, and sex or recombination is often facultative. For example, recombination occurs frequently among HIV-1 viruses (e.g. [65,91]) and, in some cases, the presence of recombinants appears to be associated with "high-risk" and/or more endemic regions. Specific recombinant genomes are also known to be associated with changes in phenotype or fitness including heightened or reduced pathogenicity or virulence [79]. In evolutionary biology, the advantage to recombination is the generation of novel gene combinations, which allows the spread of multiple beneficial mutations [30,77] and prevents the accumulation of deleterious ones [78]. Although little is known about the extent of effective recombination in many pathogenic parasitic protozoa (e.g., *Plasmodium* and *Toxoplasma*) in natural populations, it is no longer a question of whether recombination occurs, but a of question of by what form and how frequently. This combination of life history traits provides plenty of variation to study the fitness effects of recombination.

10.2 THE EVOLUTIONARY COSTS AND BENEFITS OF RECOMBINATION

The ubiquity of sex and recombination remains paradoxical, regardless of the large body of theory dedicated to the topic (for reviews of the number of alternative genetic and ecological theories to the advantages of sex, the reader is referred to [7,73,81,89]). One reason is that a number of important population genetic parameters remain unknown and, therefore, it is difficult to test evolutionary models. Estimating the strength of selection on individual sites, or haplotypes, using substitution [26,60] or population genetic model-based approaches (e.g. [11,70]) are possible. Until recently, estimates of population rates of recombination have been unobtainable.

The paradox of sex arises from the known costs associated with having sex and recombination, and whether the costs are outweighed by its potential benefits. Indeed, sex is not the most efficient form of reproduction; energy and time are required for meiosis, chiasma formations, and the production and union of differentiated gametes. For example, in *Caenorhabditis elegans*, 1416 genes are involved in syngamy, meiosis, and gamete production [88]; clearly, the time and energy associated with maintenance, transcription, and

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translation of these loci will be high relative to the cost associated with replication *via* binary fission that requires fewer loci in comparison. Additionally, there is also the well-known "2-fold of cost of sex" [97]; in a sexual species, the two individuals involved in reproduction must produce twice as many offspring as their asexual counterparts, if not, their reproductive output per individual will be lower than asexuals. Finally, there is the risk that recombining genomes, which are adapted to current environmental settings, may result in the production of deleterious combinations rather than more favorable or even, similarly fit genomes.

For an organism, the advantage of recombination can be the generation of novel gene combinations, which allows the spread of multiple beneficial mutations at multiple loci [30,77] and prevents the accumulation of deleterious genomes [78]. When mutations are beneficial, they will rise in frequency within the same population. If there are multiple beneficial mutations, the rate at which one of these beneficial mutations go to fixation is slowed, or interfered with, by other beneficial mutations in the genome (at the other loci). This interference generates selective advantages for recombining the beneficial mutations onto the same genetic background. When the mutations are on the same background, interference is removed, and the haplotype with both mutations accelerates in its rate of fixation relative to mutations that are not on the same haplotype [80]. This advantage is dependent on the mutations having additive contributions to fitness. Even among weakly selected multiple loci such as synonymous sites (and given their number in the genome), recombination appears to have considerable fitness consequences [70]. However, as Kondrashov and Kondrashov [61] demonstrated, with weak epistasis among beneficial mutations, recombination can be detrimental, as it breaks up evolved associations.

The theories associated with deleterious mutations rest on (1) the notion that recombination allows populations to escape the accumulation of deleterious mutations in small populations through a stochastic process known as "Muller's ratchet" [78], or (2) through a process which is independent of population size (mutational deterministic, MD), but requires that mutations act nonindependently (synergistic epistasis) on fitness [17,59]. Muller's ratchet is a stochastic model of the accumulation of deleterious mutations in asexual species. If the genome-wide deleterious mutation rate in an asexual population is substantial, and populations are small, then the number of individuals in the population with zero mutations will be few. *A click of the ratchet consists of the loss of individuals with the fewest deleterious mutations.* As mutations continue to accumulate over time, the distribution of mutations per individual in the population shifts; the mean number of deleterious mutations increases with every click as the least loaded class is removed with every generation. As the speed between clicks of the ratchet increases, so does the rate at which populations accumulate deleterious mutations. The speed is dependent on the size of the least-loaded class [9,18,33,40], as well other parameters, including population size, mutation rate, and the strength of selection against mutations [35,105]. For example, as the population size increases (and mutation and selection coefficients decrease), so does the time between clicks of the ratchet [35], until the ratchet is no longer an effective evolutionary force. Recombination between individuals of different genotypes slows or stops this process by shuffling mutations onto different genomic backgrounds, creating offspring with fewer or no harmful mutations. In essence, recombination regenerates the effective population size by increasing the number of alternative genotypes in the population, and in doing so, increases the effectiveness of selection against multiple deleterious mutations, removing the effects of "interference" among deleterious mutations at multiple loci [46].

Much attention has focused on the potential benefits of obligate sex in reducing the mutation load if mutations interact synergistically, for two main reasons. First, the effect is independent of population size, unlike Muller's ratchet [59]. And second, if the deleterious mutation rate (U) exceeds 1 (U needs to be >2 if stochastic factors are taken into account, 16), then obligate sexual populations can have an advantage over either facultative sexuals (organisms with both sexual and asexual phases in their life history) or asexuals [59]. Recombination returns the genetic variation lost through the fitness cost of synergistic epistasis ([16,59], see [82,89]). This process requires very little recombination, such that there is little to no increased fitness benefit in increasing the rate of recombination beyond that of having genes on multiple chromosomes [16].

Mutation rate estimates from mutation accumulation experiments (for review, see [54]), and more recent estimates from nucleotide substitution data [26,55,60] have left us currently unable to accept the MD hypothesis as an explanation for obligate sex. Estimates of U are often <1 [55]. Furthermore, experiments investigating the strength of epistasis in a number of other species such as the virus associated with foot and mouth disease (FMDV) [24], *E. coli* [25], and *C. elegans* [83] suggest that epistasis either is a very weak phenomenon or there are more complex environmental interactions that are difficult to measure in the laboratory.

10.3 EVOLUTIONARY SIGNIFICANCE OF RECOMBINATION IN PATHOGENIC MICROBES

Do the population or mutation parameters of unicellular or pathogenic species satisfy the conditions under which recombination will evolve? When are the above theories applicable to organisms such as HIV or *Plasmodium falciparum*? First, many pathogens are facultatively sexual, so the MD model is not necessarily applicable. Also, the two-fold cost of sex may not be relevant for many unicellular species. For example, given how viruses recombine, it is possible that more than two parents are involved [106], which increases the cost of sex. Second, many of these organisms replicate to an enormous population size within a host, but might undergo

extreme bottlenecks during the transmission phases of their life cycle. Furthermore, these large populations contribute to complex demography within a host, such as the striking example of HIV-1, where variability data within the host suggests a meta-population structure – a process of migration, colonization, and extinction – of viruses [31]. Population structure and bottlenecks might enhance the "ratchet" by effectively reducing population sizes; for example, Gordo and Charlesworth [35] showed that the ratchet is irrelevant for population sizes of the order of magnitude greater than 10,000. Finally, it is necessary to consider environmental fluctuations and changing selection coefficients and population sizes [15,17,66]. Fluctuating selection coefficients have dramatic effects on the number of selected alleles that go to fixation [34], and fluctuating population sizes will affect the standing variation in the population (e.g. [66]) and the fitness effects of recombination modifiers [15]. Empirical estimates of the contributing parameters to these models from natural populations of pathogens will illuminate the evolutionary potential of recombination.

Genomic recombinants are known to be associated with changes in phenotype or fitness including heightened or reduced pathogenicity or virulence. Circumstantial proof relies on the detection of genetic differences between pathogenic and nonpathogenic species (e.g. [21]). For example, strains of *Helicobacter pylori* found among patients with peptic ulcer disease and gastric cancer contain a 40-kb fragment of DNA that is not present in isolates from carriers with asymptomatic infections [21]. The discovery of this 40-kb "pathogenicity island" suggests that virulence has evolved by a single step insertion of a DNA fragment.

Direct evidence for fitness consequences of sexual reproduction has come from experiments with an RNA virus, $\phi 6$, that has proven to be an excellent model for the study of viral evolution. These phage genomes are not proofread, and as a result have extremely high rates of mutation [23,102]. Chao [13] was able to demonstrate the accumulation of deleterious mutations in small populations of this phage *via* an experiment where he subjected this virus through 40 severe bottlenecks (passaging a single phage each time), and measured their fitness (growth rate). He revealed reduced fitness values in bottlenecked lines relative to the ancestral condition, suggesting that the deleterious mutations accumulated *via* Muller's ratchet. Muller's ratchet is a population process; and although these lines are likely to have deleterious mutations, bottlenecks of one phage per generation do not necessarily replicate the stochastic process of the ratchet. Regardless, in further experiments, these different mutants were allowed to recombine, creating hybrids that had better growth rates than their parental lines [14]. They argued that the improvement in growth rate could only have arisen if (1) deleterious mutations were distributed throughout the genome and were not isolated to a particular region, and as a result, (2) were redistributed among different particles through recombination. Using larger populations, Turner and Chao [107] performed similar experiments, and demonstrated that over time, the accumulation of new beneficial mutations led to higher fitness in asexual populations. However, the fitness of sexual populations was never greater than that of the asexual populations [107]. In other viruses, anecdotal evidence of recombinants with fitness consequences exists in laboratory populations [8,32]. One example is the retrovirus associated with feline leukemia, where recombinant genotypes in the laboratory are indeed associated with increased pathogenicity [8].

Experimental studies with DNA-based microorganisms have used bacteriophage and *Saccharomyces cerevisiae*. Malmberg [67] showed that bacteriophage (T4) with high recombination frequencies adapted more rapidly to changing environmental conditions than those with lower recombination frequencies. Birdsell and Wills [10], and Grieg et al. [37] showed that specific recombinant yeast genotypes had a competitive advantage over their parental clones occasionally; but not all recombinant genotypes had a higher fitness. As a result, there is no net gain in fitness of recombining populations relative to asexuals. Finally, Zeyl and Bell [112] showed that recombining populations of *S. cerevisiae* did not have a selective advantage over non-recombining populations unless both the recombining and the non-recombining populations were kept on ancestral rather than new environments.

For *Toxoplasma gondii*, the parasite populations can be clustered into three distinct clonal lines globally, although recombinants are found but at low frequency (references). The three strains vary in their degree of virulence, with variation being limited to within two of the three lines, and the most prevalence strain also being the most virulent. This overall lack of variation is confirmed by studying genetic polymorphism of immunogenic loci, which would likely be sources of polymorphism associated with escaping host immunity. Grigg et al. [38] have argued that a number of genotypes have arisen from the "mixing" of two of the three lines, although no test of recombination was employed. Recently, crossing experiments have revealed recombinant genotypes that appeared to be associated with much higher virulence (references, David Sibley).

Recombination contributes to the genetic variation in antigenic determinants in parasitic protozoa, including malaria parasites, coccidia and trypanosomes. *Plasmodium* and *Trypanosoma* have many variant antigen genes (var genes in *P. falicparum* and VSG genes in trypanosome), or genes that encode surface proteins such as merozoite (MSP) and circumsporozoite (CSP) proteins, suggesting a role for genetic exchange in enhancing parasite diversity. Malaria parasites are hermaphroditic and haploid for most of their life cycle, with asexual replication in the primate host and zygote formation and meiosis occurring during the mosquito phase of development. Haploid parasites divide mitotically, and some cells differentiate into male and female stages. Male and female gametes fuse in the mosquito host to form a short-lived diploid zygote. Meiotic division then gives rise to haploid cells that develop into infective sporozoites, which migrate to

the mosquito salivary glands and infect humans during mosquito blood-feeding. In populations where endemicity is low, fusion of male and female gametes from the same clone (selfing) is more likely and results in no effective recombination, whereas in populations with high endemicity, individual hosts are frequently infected with multiple genotypes, and fusion of gametes from different clones (outcrossing) may result in recombination.

The extent to which *P. falciparum* is effectively clonal in nature has been the subject of debate. Relatively low diversity, especially at synonymous and intron sites, and lack of LD within some genes (*csp*) have led some authors to suggest that *P. falciparum* has recently suffered a dramatic reduction in population size followed by a recent mass global expansion [6]. Corroborating evidence comes from single-nucleotide polymorphism (SNP) analyses of introns in a number of loci, revealing little to no variation from a sample of eight clones scattered globally, but mainly from Papua New Guinea [41,108]. However, Hey [44] has argued that synonymous substitutions between species also appear to be uncommonly "restricted" in malaria (the genome exhibits 80% AT composition), suggesting that biased mutation rates, not just population dynamics, are affecting the accumulation of synonymous and intron variation.

10.4 RECOMBINATION AND ITS EFFECTS ON EVOLUTIONARY INFERENCES WITHIN A SPECIES

Evolutionary histories of genomic loci or sites are correlated depending on the rate of recombination between regions (Fig. 10.1). When recombination occurs, no single phylogeny can describe the ancestry of a length of nucleotide sequence because different parts of the nucleotide sequence will then have separate, but correlated, histories. Recombination generates degrees of independence by breaking up the linkage relationships of mutations at different sites that have evolved over time. The extent or rate of genetic rearrangement *via* recombination in natural populations is crucial if we are to use genome and genetic mapping information to locate genes that underlie important phenotypes (e.g., genes associated with virulence, transmission, and immune evasion). In medical genetics, associations between disease phenotypes and genetic markers built up through genetic drift and broken down by recombination are central to the mapping of disease-associated mutations [63,86]. Mutations at distinct loci or sites may occur on either the same or different genetic "backgrounds." Recombination shuffles those mutations and backgrounds. Recombination reduces the variance of population genetic parameters and test statistics of natural selection (e.g., Tajima's *D*) by generating independence among the evolutionary histories of physically linked genomic regions. This is an advantage because different parts of the sequence represent different, although correlated, realizations of the evolutionary process. Each realization is associated with a large variance, but together, the variance is reduced if independent.

The occurrence of recombination also complicates analysis. Although epidemiologists use phylogenies to make inferences about routes of disease transmission for various pathogens and to estimate evolutionary parameters (e.g., rates of molecular clocks, rate heterogeneity, mutation bias, or selection) or demographic processes [3,31,43,49,62,87, 92,93,111], how recombination affects these various phylogenetic approaches in estimating evolutionary parameters remains largely untested (but see [84,95]). Nevertheless, it seems clear that only when recombination is absent are direct inferences from phylogenies likely to be reliable.

A very useful tool for population genetics is a probabilistic genealogical model known as the coalescent. Extensions of the coalescent model can be used to study alternative demographic models such as population expansion, as well as recombination. The coalescent is a statistical genealogical description of a set of alleles, haplotypes, or sequences sampled randomly from a population [51,57,58], and also provides a framework in which properties of populations can be estimated, such as drift,

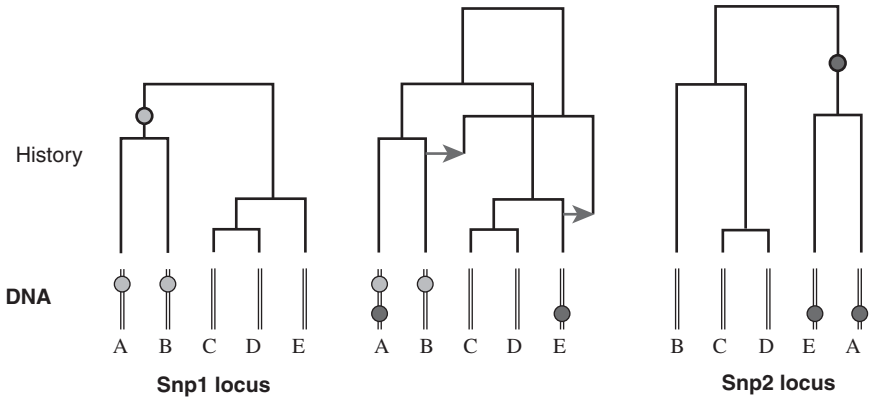


Fig. 10.1. The effect of recombination on gene genealogies at physically linked sites. Recombination allows for individual segregating sites, or SNPs as are shown here, to have independent evolutionary histories. The history in the middle is an ancestral recombination graph integrating the two histories. See color plates.

mutation, and recombination. Because the genealogy is unknown, it is treated as an unknown parameter and a number of potential genealogies exist for a dataset, unlike a phylogeny where a single history is assumed. A computer simulation of a coalescent can be performed by drawing a random genealogy for a given sample of sequences, with lineages coalescing going backwards in time, until all alleles have coalesced. The point in time where all alleles have coalesced is known as the mean time (across genealogies) to the most recent common ancestor (MRCA). The rate that samples or branches coalesce within the genealogy depends on the size of the sample and the size of the population. The more individuals sampled, the faster the rate of coalescence; in contrast, the larger the population, the more potential coalescent events, and the slower the rate. Neutral mutations can be placed randomly onto branches of the genealogy at a constant rate. If mutations occur at a constant rate, then larger genealogies will have a greater number of observed mutations than the expected number of mutations, and in this way, it can be seen how the MRCA of all sampled alleles from a single population is directly related to the level of polymorphism observed in the population. Similarly for recombination, if events are uniformly distributed across a chromosome, and Poisson distributed over time, the number of recombination events that are observed in a population are also dependent on the time to the MRCA. Recombination creates a network of relationships in the genealogy (also known as the ancestral recombination graph, Fig. 10.3), with mutations traceable throughout the genealogy, but increasing the overall time to the MRCA. Finally, the effects of recombination on sample history are a function not of the absolute recombination rate but of the product of the per gene per generation rate of crossing over (genetic map length), r, and the effective population size, N_e. Without prior information about one of these parameters, it is possible only to estimate the product of these parameters.

For a random neutral genealogy, there is an expected distribution of branch lengths [96]. Many terminal branch lengths will be short, implying shorter times until a common ancestor for that pair of sequences and within the tree, branch lengths are longer, implying a smaller probability that a pair of ancestors share a common ancestor further back in time. Empirically, when terminal branches (tips of the tree) appear very long, this pattern can be interpreted as evidence for population expansion [48,96]. However, population geneticists [49] have known for some time that recombination has a similar effect on gene trees because recombination makes sequences more homogeneous, thus creating more star-like trees (with longer terminal branches). The mean diversity between pairs of sequences will be the same because the number of mutations does not differ significantly, and the variance in pairwise distances decreases with increasing recombination [49]. In the absence of information about recombination, it is therefore difficult to separate the effects of demographic explanations on a given star-like tree from those of recombination.

The genealogical affects of multiple or recurrent mutations occurring at a single site are also difficult to separate from recombination. In a non-recombining environment, homoplasies (instances of parallel evolution) on a tree are taken for granted to be the result of multiple or recurrent mutation at a site. However, recombination also generates homoplasies by moving mutations onto different backgrounds. It is well known that unicellular taxa have higher per base mutation rates than multicellular taxa [22,23]. Therefore, the probability of recurrent mutations is higher in these taxa. It is therefore necessary to address this possibility when assessing the contributing factors to observed genealogical relationships.

10.5 DETECTING AND ESTIMATING RECOMBINATION

All approaches to detect or estimate the frequency of recombination, either parametric or nonparametric [4], are dependent on an assumed underlying mutation model and some assumptions about demographic processes, although some are more robust to deviations than others. Regardless, to test population genetic models of pathogens, estimates of the recombination rate, and mutation rates, are vital. The following outlines a few phylogenetic and population genetic methods to detect recombination and estimate population rates of recombination. We discuss the relevance of recombination events that are associated with changes in fitness and pathogenicity in selected taxa.

For many unicellular pathogens, observed levels of nucleotide polymorphism in many populations indicate high mutation rates [22,23] that makes inference of recombination difficult. For example, both mutation and recombination contribute to haplotype variation at linked sites. High mutation rates can hide haplotype structures and recurrent mutation events at a single site may be attributed to recombination. Consequently, a large number of nonparametric and parametric methods have been developed to detect the presence or absence of recombination with the possibility of these mutation events in mind. The nonparametric methods can be considered as belonging to two main categories – comparative and phylogenetic (reviewed in [4]). Parametric methods are model based and deal specifically with haplotype structure – or LD, in population genetic datasets.

10.5.1 Nonparametric Analyses

Comparative nonparametric approaches statistically evaluate genomic characteristics such as base identity, codon usage, and base composition among separate taxa. Other nonparametric methods (see [4]) employ some property of phylogenetics. In principle, these methods are similar to identifying phylogenetic reticulations or incongruencies between gene trees of different species. Recombination is implicated when regions or genes have different phylogenetic histories [39,36,69], or through assessing properties of trees inferred under the assumption of no recombination [99,110]. All of these methods vary in their ability to detect recombination. Recently, Posada and Crandall [85] assessed *via* simulations 14 different nonparametric methods to detect recombination.

Phylogenetic analyses of alleles from housekeeping genes of *Neisseria*, *Staphylococcus*, *Campylobacter*, *E. coli*, *Salmonella*, and *Helicobacter* species (e.g. [29]) demonstrate the relevance of recombination to the evolutionary history of these species. Multilocus genotypes have been used extensively to investigate the genetic structure of bacterial pathogens in the past 20 years [12,28,42,68,100,103). The statistical evidence for recombination in many of these analyses often comes from reticulations, incongruencies, or homoplasies inferred from phylogenetic-based treatments of the data. In particular, this approach demonstrated that although some bacteria, such as *Neisseria gonnhorrea*, show high levels of recombination, others, such as *E. coli* and *Salmonella*, have a predominantly clonal population structure. It has been argued that in *Neisseria*, recombination generates a pool of variation from which resistant clones may arise and proliferate, having evolved to a specific ecological niche (e.g. [27,68]). However, the clonal propagation of a successful clone or haplotype will contribute to extensive LD [10], and may obscure the frequency with which recombination actually occurs.

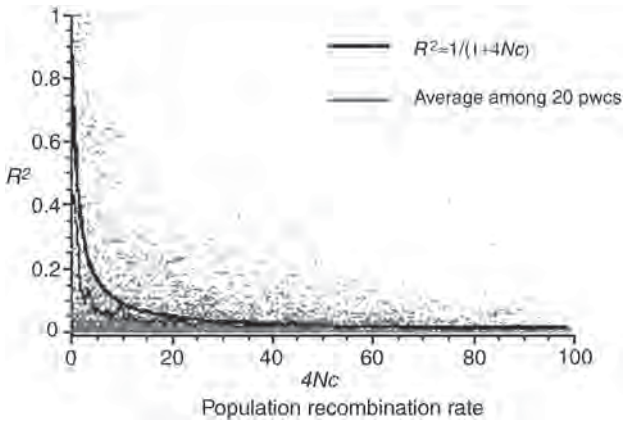


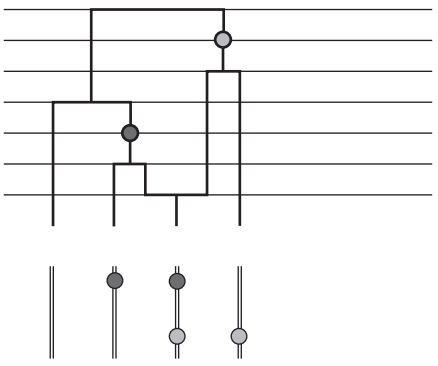
Fig. 10.2. The relationship between LD and recombination/physical distance. Plotted are the pairwise estimate of a measure of LD, R^2, which is a measure of cavaration between pairs of sites, and the recombination distance ($4Nr$), where N is the effective population size and r is the rate of recombination, between the pairs of sites compared. One hundred segregating sties are shown. The theoretical relationship between the decay of LD and recombination rates are shown.

10.5.2 Parametric Methods

10.5.2.1 LD, the coalescent, and 4Nr In order to estimate population recombination rates, a population model that addresses the information embedded in haplotype variation is required. A number of parametric methods are available that are independent of any inferred phylogeny and utilize summary statistics of associations between segregating sites to detect recombination events. LD, the nonrandom association of alleles at linked sites, is a fundamental aspect of genetic variation. The more distant two sites are from each other, the greater the probability that a recombination event will occur between them; recombination creates a decay of LD between nucleotide sites (Fig. 10.2). How LD decays with respect to the physical distance between sites is both an analytical and a theoretical description of how recombination breaks up pairwise associations, and is often used as a test of recombination [5,20,71,74,94].

The main advantage of LD-based methods over phylogenetic methods is that LD statistics have direct relationships both with the data and with the population genetic theory [46,64]. As a result, LD can be used with model-based (parametric) approaches to estimate the rate of recombination [45,50,52,71]. However, a simplification of the population history of a sample, and the mutation and recombination models, are almost always required for these models when estimating population parameters.

Hudson [52] developed a method for estimating the population recombination rate by using the coalescent. Given a likelihood based on a coalescent model with recombination for each haplotype configuration (Fig. 10.3), for all pairwise comparisons of segregating sites, he combined the likelihoods to approximate the population recombination rate. The composite likelihood estimate (CLE) of the recombination rate is



Event	Probability
Coalescence	$1 / (\rho + 1 + \theta)$
Mutation	$\theta / (4\rho + 4 + 4\theta)$
Coalescence	$1 / (\rho + 3 + 5\theta/4)$
Coalescence	$1 / (3\rho/2 + 6 + 7\theta/4)$
Mutation	$\theta / (6\rho + 24 + 7\theta/4)$
Coalescence	$1 / (3\rho/2 + 10 + 2\theta)$
Recombination	$\rho / (4\rho + 10 + 4\theta)$

$\theta = 4N_e\mu$ $\rho = 4N_e r$

Fig. 10.3. Coalescent modeling of recombination. The coalescent is a "backwards" process for modeling genealogies. Parameters of critical interest, such as mutation and recombination, can be mapped onto these genealogies. In this way, we can see how both the genealogical history and recombination affect the pattern of polymorphism among sites in the genome. See color plates.

the estimate associated with the largest sum of probabilities over all pairs of sites. Because the method sums over all pairwise comparisons, it introduces nonindependence, and as a result, the true variance of single estimate of the CLE recombination rate [52] is unknown without simulation [72]. The CLE method is flexible and potentially can be expanded to incorporate deviations from the standard coalescent. An extension of Hudson's [52] CLE estimator is the relaxation of the infinite sites model assumptions, allowing for recurrent mutation and recombination rate variation among sites across a region. Relaxation of the infinite sites model is necessary for many unicellular genomes because of high mutation rates.

The biological conditions under which many pathogens have sex plays a role in the population recombination estimate obtained. It is clear that endemicity is directly related to heterozygosity and recombination frequencies in natural populations of protozoans such as *P. falciparum* (e.g. [1,2]). Effective recombination among various parasites such as those causing malaria or Chagas' disease depends on the presence of two different genotypes in the hosts during the sexual phase of development. As a result, population recombination rates may vary dramatically depending on endemicity at sampling locations. However, for many protozoans, actual recombination rates inferred from genetic maps are relatively large when compared to other taxa with facultative sex.

Recently, both parametric and nonparametric methods were used to infer recombination rate variation in four separate *P. falciparum* population samples [75]. To study the nature and scale of LD and recombination variation in the parasite genome, Mu et al. (2005) assayed 99 worldwide *P. falciparum* isolates and one chimpanzee parasite *Plasmodium reichenowi* for SNP spanning chromosome (Chr) 3 at an average interval of one SNP per ~5.5 kb. They showed high variation in recombination rate among different parasite populations and along Chr 3 of the parasite (Table 10.1). The majority of recombination events cluster near the chromosome ends and in the middle of the chromosome (Fig. 10.4). Nonparametric estimates revealed many recombination events as well as recombination hotspots among African parasites. Similar recombination hotspots were also found in the remaining parasite populations except America where the hotspot in the middle of the chromosome is absent. Parametric methods, based on coalescent models, also detected significant recombination rate variation in all populations except Africa, for which the high levels of historic recombination invalidated the test. Figure 10.4 shows the recombination map along the chromosome for the four populations, as estimated by the Reversible Jump Markov Chain Monte Carlo (RJMCMC) method [71,72]. Although the sample size for PNG is fairly small and may account for the discrepancy between the nonparametric and parametric estimates (Table 10.1), the inferred location of recombination events using the two approaches generally concur.

Although the overall population recombination rate is highly variable among populations, the chromosomal locations of major recombination hotspots were conserved. Telomeric regions in *P. falciparum* clearly exhibit elevated crossing over. The conservation is likely due in part to the shared evolutionary ancestry of *P. falciparum* populations. Additionally, these regions contain a high density of genes such as *var*, *rifin*, and *stevor*, whose products are implicated in cell–surface interactions and are consequently under strong immune-mediated diversifying selection (as demonstrated by the high rate of amino acid evolution). These observations suggest elevated recombination rates may play a significant role in generating multiple haplotypes at genes important for *P. falciparum*'s evasion of host immunity.

Recently, Mu et al. [76] analyzed 204 genes on Chr 3 of *P. falciparum*, revealing more extensive polymorphism than previously observed, identifying 238 SNPs and 165 microsatellites. Synonymous variation was clearly reduced, but the predominance of nonsynonymous segregating sites suggests that the base composition is likely affecting synonymous (codon usage) and intron variation [76], perhaps accounting for the discrepancy between the large-scale SNP survey and the previous studies. The estimate of the effective population size of malaria from the Chr 3 survey is over 300,000, as previously reported by Hughes and Verra [53]. The time to the most recent common ancestor was estimated to greater than 100,000 years ago, 2 orders of magnitude greater than the estimate previously reported [90].

Conway et al. [20] showed that LD broke down readily at the gene encoding merozoite surface protein-1 (*msp1*) from

TABLE 10.1. Relative Rates of Genetic Drift and Recombination in Four Different Population Samples of *P. falciparum* for Chr 3 (from [75])

Population	Sample size for each population	Relative rates of genetic drift $[N_e/(1 + f)]^a$	Relative rates of recombination $[N_e(1 - f)]^b$
Africa	36	1.02	>100
America	23	1.00	1.00
SE Asia	29	0.73	2.43
PNG	11	0.75	11.9

f is the inbreeding coefficient.

[a] $N_e/(1 + f)$, relative compound population parameter inferred from genetic drift rates (Nicholsen et al., 2001; Marchini et al., 2004).

[b] $N_e(1 - f)$, relative compound population parameter inferred from recombination rates.

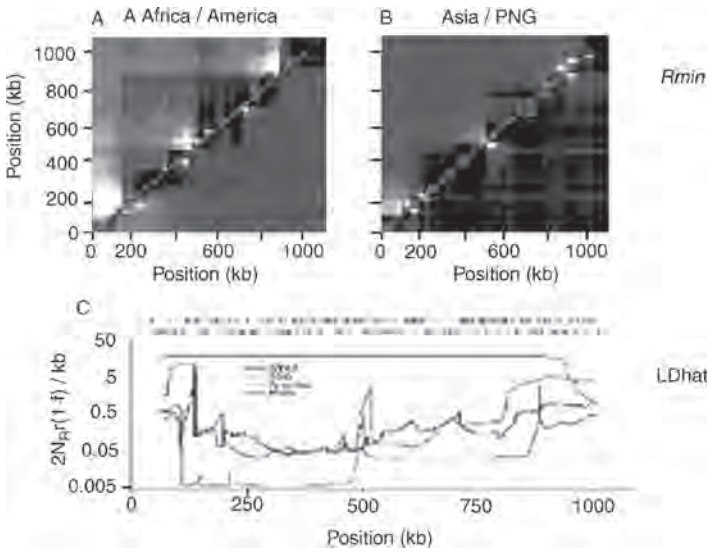


Fig. 10.4. Hotspots of recombination in four populations of *P. falciparum* [75]. The two top panels are estimates of the minimum number of recombination events, for each of four different populations, for all pairs of sites obtained using the method of Myers and Griffiths (2003) with the program Recmin. The bottom panel shows parametric estimates of population recombination rate variation across the third chromosome using the program LDhat. See color plates.

samples taken from populations in Africa, suggesting extensive recombination among these parasites in some populations. Anderson et al. [1,2] examined LD among 12 microsatellite loci within each of nine populations, three each from Africa, South America, and Asia, revealing considerable global variation in the amount of LD among loci within and between populations. It was clear that populations with higher variation, and higher endemicity, had the least amount of LD (especially in Uganda and Democratic Republic of Congo). The high rates of recombination observed in some populations may make it difficult to use association mapping approaches to find loci involved in host immunity, because recombination will break down linkage relationships between target and marker loci, even when a marker is physically close to a candidate locus [1,2].

One strategy that a pathogen might use to evade host immunity is to have a greater arsenal of variation at antigenic sites in the population's repertoire. Selection for maintaining elevated levels of genetic variation – "balancing selection" – might be an important evolutionary force contributing to a pathogen's ability to evade hosts immune systems. Conway et al. [19] identified a region of the MSP1 gene in *P. falciparum* that appears to be associated with immunogenicity by examining the polymorphism at different sites along the MSP1 gene and testing whether these individual regions deviated from the neutral expectation of Hardy–Weinberg equilibrium. Alleles were sampled from different populations; and under neutral expectations and depending on the degree of isolation between populations (migration), there should be some degree of differentiation between different populations. Those regions exhibiting less differentiation than others might be subject to selection.

Critical to this analysis is the action of recombination generating independence among sites within the gene. Recombination not only contributes to the overall variation, at the Msp1 locus but is also necessary for the identification of important regions that are under selection. For example, if all sites were completely linked, then they might exhibit the same population variation due to linkage. A site that is under balancing selection will affect linked variants by maintaining them in the population, and other neutral sites nearby will also exhibit increased levels of variation. In contrast, beneficial mutations that sweep through the population will drag linked alleles with them (hitchhiking) depending on the rate of recombination between the sites. This action contributes to reduced variation around the beneficial mutation [98]. Such reduced variation is a signal of the action of selection [56]. One example is the observed high LD near the chloroquine resistance gene, *pfcrt*, which is consistent with recent directional selection of Wootton et al. [109].

10.6 CONCLUSIONS

Recombination occurs at substantial frequencies in natural populations of many pathogenic species and plays a significant role in pathogen evolution and their ability to adapt to changing environments. Although it is still uncertain whether the fitness of obligately sexual species is greater than that of asexuals or facultative asexuals, it is clear that taxa with a sexual life

cycle maintains an alternative mechanism other than mutation, to generate novel multilocus genotypes.

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CHAPTER 11

Evolutionary History of the Malaria Parasites

Francisco J. Ayala

Department of Ecology and Evolutionary Biology, University of California Irvine, CA 92697, USA

11.1 MALARIA'S HUMAN TOLL

Malaria is caused by species of *Plasmodium*, a parasitic protozoan. Four species of *Plasmodium* are parasitic to humans: *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*. *P. falciparum* is the most pervasive and malignant human malarial parasite. It causes 300–500 million cases of clinical illness per year and 1.5–2.7 million deaths in Sub-Saharan Africa, plus 5–20 million clinical cases and 100,000 deaths elsewhere in the world, 80% of them in Asia [84]. Most geographically widespread and prevalent in many regions outside Africa is *P. vivax*, which accounts annually for 70–80 million clinical cases across much of Asia, Central and South America, the Middle East, and Africa. The human toll exacted by *P. malaria* and *P. ovale* is small by comparison, although still substantial. Hippocrates (460–377 B.C.), the father of Greek medicine, describes chronic tertian and quartan fevers, which were surely forms of malarial disease, as his description makes it clear: "Those who drink [stagnant water, which he thought responsible for malarial symptoms] have always large, stiff spleens and hard, thin, hot stomachs, while their shoulders, collar bones and faces are emaciated; the fact is that their flesh dissolves to feed the spleen" (cited by [62, p. 91]). In the ancient Mediterranean region, Malaria may have, indeed, inhibited agricultural expansion and restricted population growth [10]. In the Roman Compagna and other parts of Italy, the Romans dug elaborate underground channels to drain swamps and secure water for irrigation and drinking, which may have largely prevented malaria. After the fall of Rome, through the Middle Ages and until modern times, malaria depopulated Italy as well as other parts of the Mediterranean region ([41] and [62, pp. 89–92]). Malaria was finally eradicated from southeast Spain, the Roman Compagna, parts of Greece, and elsewhere in southern Europe, only a few years after the Second World War. According to Frank Ashall [2, chapter 13], throughout human history malaria has killed more soldiers than have died as a result of war. During the US Civil War, more than half of all soldiers on both sides of the conflict became infected with malaria.

Malaria is a reemerging disease in several countries and an expanding malady in Sub-Saharan Africa, where malaria's toll may have doubled over the last two decades. Moreover, malaria is becoming a health problem in Western Europe: about 6500 cases are reported annually in Germany, France, Italy, and the United Kingdom, 64% caused by *P. falciparum* and 23% by *P. vivax*.

11.2 EVOLUTIONARY ORIGINS OF *PLASMODIUM*

The genus *Plasmodium* consists of nearly 200 named species that parasitize reptiles, birds, and mammals. *Plasmodium* belongs to the Apicomplexa, a large and complex phylum with about 5000 known species and as many as 60,000 yet to be described [20,28,52, pp. 1–21] and [86]. The Apicomplexa are all parasites, characterized by the eponym structure, the apical complex. The taxonomy and phylogeny of the phylum have been the subject of controversy and frequent revision. One issue is whether the genus *Plasmodium* evolved directly from monogenetic (i.e., single host) parasites of the ancient marine invertebrates from which the chordates evolved, or whether it originated by lateral transfer from other, already digenetic (i.e., two hosts), parasites of which one would have been a vertebrate [8,37,44,56,58]. There is no fossil record of apicomplexans [57], but molecular investigations indicate that the phylum is very ancient, perhaps as old as the multicellular kingdoms of plants, fungi, and animals, and thus somewhat older than 1 billion years [5,28] (Fig. 11.1).

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

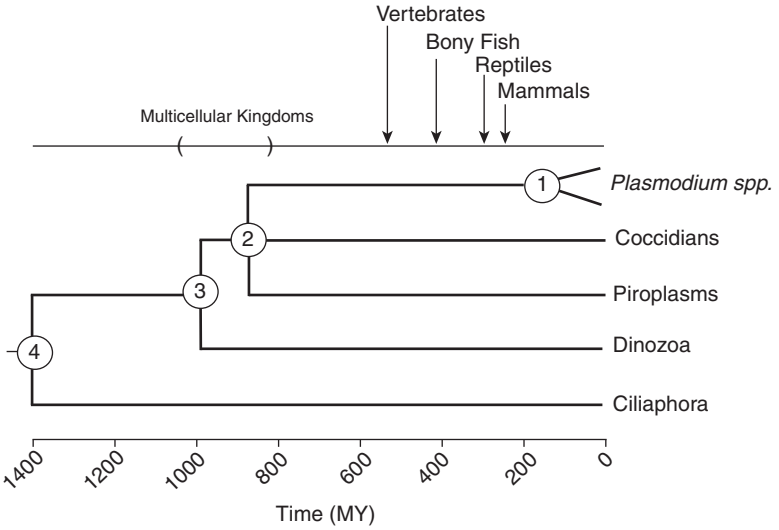

Fig. 11.1. Simplified phylogeny of *Plasmodium* and related protozoa. The branching nodes refer to (1) radiation of the *Plasmodium* genus; (2) radiation of the phylum Apicomplexa; (3 and 4) Apicomplexa divergence from two related phyla, Dinozoa and Ciliophora.

Coccidians and piroplasms are two orders of Apicomplexa related to Plasmodium. The Coccidea include species parasitic to mollusks and marine annelids, but many are parasitic to mammals and other vertebrates [86]. Many coccidians, such as *Cryptosporidium*, are monogenetic parasites, able to complete their life cycle within one single host. But other coccidians, including *Neospora*, *Sarcocystis*, and *Toxoplasma* are digenetic: their complete development requires two successive hosts. The Piroplasmida, as well as the Haemosporida, the order to which *Plasmodium* belongs, are digenetic parasites: maturation of gametes, fertilization, and sporogony occur in the hematophagous invertebrate vector; the rest of the life cycle is completed in the blood of a vertebrate. The Dinozoa are the phylum most closely related to the Apicomplexa. The Apicomplexa/Dinozoa clade may have originated about the time or earlier than the origin of the multicellular kingdoms [28].

Various molecular phylogenetic analyses have revealed the relationships among the *Plasmodium* species. Figure 11.2 shows a representative phylogenetic tree based on the circumsporozoite

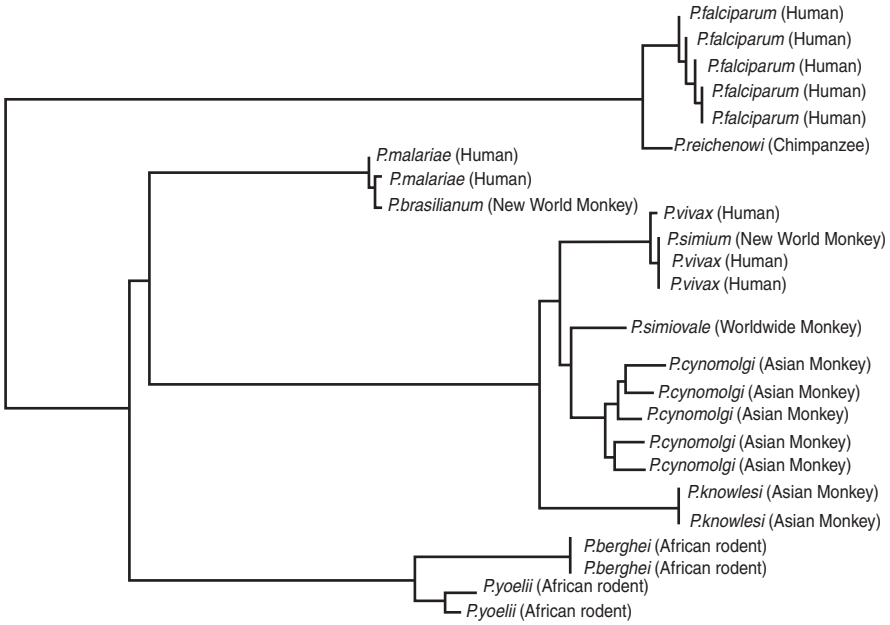

Fig. 11.2. Phylogeny of 11 *Plasmodium* species (30 isolates) inferred from *Csp* gene sequences. Each parasite's host is given in parentheses. Some independent isolates have identical sequences of which only one is shown; thus, only five out of eight isolates are shown for *P. falciparum*, three out of four for *P. vivax*, and one out of two for *P. simium*.

TABLE 11.1. *Plasmodium* Species Used for Construction of Phylogeny Based on *Csp* Sequences

Species	Number of Strains	Host	Geographic Distribution
P. falciparum	8	Human	Tropics worldwide
P. malariae	2	Human	Tropics worldwide
P. vivax	4	Human	Tropics worldwide
P. reichenowi	1	Chimpanzee	African tropics
P. brasilianum	1	Monkey	New World tropics
P. simiovale	1	Monkey	Tropics worldwide
P. cynomolgi	5	Monkey	Asian tropics
P. simium	2	Monkey	New World tropics
P. knowlesi	2	Monkey	Asian tropics
P. berghei	2	Rodent	African tropics
P. yoelii	2	Rodent	Africa

protein (CSP) gene sequences. Table 11.1 provides information about the hosts and the geographic distribution of the species. The tree is obtained by the "neighbor-joining" method (NJ) [79] based on genetic distances calculated according to Tamura's three-parameter method [82]. Trees obtained with other methods (such as maximum likelihood), and/or based on other measures of genetic distance, have fundamentally identical topologies as those shown in Figure 11.2 (certainly with respect to the conclusions that will be formulated below). (Additional details can be found in [5,28,29]. Estimates of divergence times based on two genes, *Csp* and *rRNA* trees are given in Table 11.2.

The phylogenies represented in Figure 11.2 include three human parasites, *P. falciparum*, *P. vivax*, and *P. malariae*. All four species parasitic to humans (i.e., including *P. ovale*) have been included in phylogenies based on other genes, such as the mitochondrial gene encoding cytochrome b [67,69]. The phylogenetic relationships among the human parasites are fundamentally identical to those shown in Figure 11.2, with *P. ovale* closer to *P. vivax* than to *P. falciparum* or *P. malariae*.

These phylogenetic studies yield the following conclusions concerning the evolutionary history of the human malarial parasites:

1. The four human parasites, *P. falciparum*, *P. ovale*, *P. malariae*, and *P. vivax* are very remotely related to each other, so that the evolutionary divergence of these four human parasites greatly predates the origin of the hominids 8 million years ago. It follows that their parasitic associations with humans are phylogenetically independent; that is, all but one (at the most) of these species has been laterally transmitted to the human ancestral lineage from other, non-primate hosts. These results are consistent with the diversity of physiological and epidemiological characteristics of these four *Plasmodium* species [13,53].
2. *Plasmodium falciparum* is more closely related to *Plasmodium reichenowi*, the chimpanzee parasite, than to any other *Plasmodium* species. The time of divergence between these two *Plasmodium* species is estimated at 8–11 million years (My) ago, which is consistent with the time of divergence between the two host species, human and chimpanzee. (The divergence time of parasitic species is likely to predate the divergence of their host species, similarly as the divergence times of ancestral gene lineages are likely to predate the divergence of their species; alternative polymorphic states may become fixed in one or the other carrying species.) A parsimonious interpretation of this state of affairs is that *P. falciparum* is an ancient human parasite, associated with our ancestors since the divergence of the hominids from the great apes.

TABLE 11.2. Time of Divergence (in Million Years) Between *Plasmodium* Species, Based on Genetic Distances at Two Loci (Ref. 6)

	rRNA	Csp
falciparum vs. *reichenowi*	11.2 ± 2.5	8.9 ± 0.4
vivax vs. monkey[a]	20.9 ± 3.8	25.2 ± 2.1
vivax vs. *malariae*	75.7 ± 8.8	103.5 ± 0.6
falciparum vs. *vivax/malariae*	75.7 ± 8.8	165.4 ± 1.6

[a] *brasilianum* and *simium* not included.

Some authors (e.g. [61]) have failed to separate unambiguously *P. falciparum* and *P. reichenowi* when they analyzed amino acid rather than nucleotide sequences. This ambiguity can be attributed to the difficulty of aligning, for several *Plasmodium* species, amino acid sequences, such as CSP, that are quite different and variable in length ([29], see also [76], with the consequence that only the more conserved amino acids can be reliably aligned. When suitable comparisons are made between *P. reichenowi* and *P. falciparum*, the difference between the two species is unambiguous (see [76] for the distinct composition of the central repeat region of *Csp* genes).

3. *P. malariae*, a human parasite, and *Plasmodium brasilianum*, a New World monkey parasite, are genetically indistinguishable at the *Csp* gene. We infer that a lateral transfer between hosts (a "host switch") has occurred in recent

TABLE 11.3. Average Genetic Distance Within and Between various *Plasmodium* Species, Based on the *Csp* Gene

Species	Number of Strains	Intraspecific	Interspecific			
			malariae	*vivax*	*simium*	*brasilianum*
falciparum	8	0.009±0.001	0.697±0.003	0.581±0.003	0.837±0.002	0.687±0.004
malariae	2	0.004±0.003	—	0.517±0.006	0.513±0.004	0.002±0.002
vivax	4	0.004±0.001	—	—	0.004±0.001	0.517±0.000
simium	2	0.000±0.000	—	—	—	0.508±0.187

times, either from monkeys to humans or *vice versa*. A host switch is defined as a shift of a parasite from one host species to another distantly related host species.

Moreover, *P. vivax* is genetically indistinguishable from *Plasmodium simium* at the *Csp* gene (as well as at numerous other genes; see [30,50,53]). *P. simium* is, like *P. brasilianum*, a parasite of New World monkeys. We infer, again, a recent lateral transfer between human and monkey hosts.

The average intraspecific distances for each of the three human parasites (and for *P. simium*; only one strain of *P. brasilianum* was investigated) are shown in Table 11.3. The table also gives the genetic distance between the three human and the two primate parasites. The genetic distance (nucleotide substitutions per site) between *malariae* and *brasilianum* is 0.002 ± 0.002, not greater than the distance between the two *P. malariae*, or the four *P. vivax*, or the eight *P. falciparum* sequences available, although *P. malariae* and *P. brasilianum* are isolated from very different hosts – *P. malariae* from humans, *P. brasilianum* from New World monkeys. The question arises whether they are different species, because they are genetically indistinguishable. *P. malariae* and *P. brasilianum* might be considered either two distinct species or a single species exhibiting "host polymorphism" [27]; that is, they are able to parasitize more than one host species. A similar issue might be put forth with respect to *P. vivax* and *P. simium*, because these two are also genetically indistinguishable (genetic distance 0.004 ± 0.001; Table 11.3).

11.3 HUMAN TO MONKEY OR MONKEY TO HUMAN?

Whether or not the two species in each human–primate parasite pair (*P. vivax*–*P. simium* and *P. malariae*–*P. brasilianum*) should be considered the same or distinct species is merely a matter of taxonomy and nomenclatural convenience, rather than biologically substantive. What is important is the conclusion that two of the four known human malaria parasites have each nearly identical platyrrhine (New World monkey) parasite relatives. This is a strong indication that a host switch has occurred in recent times (and may continue to occur). This is in stark contrast to the observed relationship of *P. falciparum* and *P. reichenowi* that have evolved *vertically* (i.e., from a common ancestor), in parallel with their respective human and chimpanzee host lineages.

Determining the direction of the host switch between human and platyrrhine – either from monkey to human, or human to monkey – holds great biological relevance for understanding the evolution of the genus and understanding the origins of the disease. Humans and platyrrhine monkeys are distantly related (their most recent common ancestor lived some 35–40 million years ago) and have been geographically associated only after the first human colonization of the Americas, which occurred within the last 15,000 years. Indeed, the host switch may have occurred following the second influx of humans after the European colonizations of America in the sixteenth century. Whether 500 or 15,000 years have passed since the host switch, it would be a mere moment in evolutionary time, and so it is not surprising that the human and platyrrhine parasites are genetically so little diverged. Both *P. simium* and *P. brasilianum* are known to be infectious to humans [38]. Epidemiological serosurveys of humans and monkeys in French Guiana indicate that platyrrhines may actually serve as zoonotic reservoirs for human disease [35], thus lending support to the host–polymorphism hypothesis.

Unlike *P. reichenowi*, which thrives exclusively in chimpanzees and perhaps gorillas, the platyrrhine malaria parasites are quite capacious in their host preference, so that these New World parasites appear quite susceptible to host switches. *P. simium* infects at least three, and *P. brasilianum* as many as 26 species of New World monkeys [40]. We have argued in the past on the grounds of evolutionary parsimony, that the possibility should be considered that the host switches observed in *P. vivax/simium* and *P. malariae/brasilianum* may have occurred from primates to humans rather than simply assuming the opposite [29,75].

Several considerations favor a lateral transfer from human to monkey hosts:

(i) *P. vivax* has a worldwide distribution, in contrast to the limited geographic range of *P. simium*, which is restricted to a few South American monkey species, *Alouatta fusca*, *Brachyteles arachnoides*, and *Ateles* sp. [40]. The counterpoint can be made, however, that humans are exceedingly

mobile. Infected humans could readily have carried the parasite from South America to other continents.

(ii) There are no records of malaria in South America (or elsewhere in the New World) before the arrival of the European colonizers within the last 500 years. This would be consistent with the interpretation that *P. vivax* (as well as *P. malariae* and *P. falciparum*) was introduced to the New World by the European colonizers and their African slaves. The weakness of this argument is that it consists of negative evidence, which is particularly unreliable when there are no extensive observations, experiments, or health records that would have likely manifested the presence of malaria in the New World before the year 1500, even if malaria had indeed been present.

(iii) Historical records suggest that nonmalignant malaria has occurred in the Old World for several thousand years. Chinese medical writings, dated 2700 B.C.; cuneiform clay tablets from Mesopotamia, dated about 2000 B.C.; the Ebere Egyptian Papyrus (*ca.* 1570 B.C.); and Vedic period Indian writings (1500–800 B.C.) mention severe periodic fevers, spleen enlargement and other symptoms suggestive of malaria [80]. Spleen enlargement and malaria antigen have been detected in Egyptian mummies, some more than 3000 years old [63,80]. Hippocrates' (460–370 B.C.) discussion of tertian and quartan fevers, "leaves little doubt that by the fifth century B.C. *Plasmodium malariae* and *P. vivax* were present in Greece" [80, p. 3]. If this interpretation is correct, the association of *P. malariae* and *P. vivax* with humans could not be attributed to a host switch from monkeys to humans that would have occurred after the European colonizations of the Americas. This seems definitive evidence, so long as one accepts the interpretation that the fevers described by Hippocrates were indeed caused by the two particular species *P. vivax* and *P. malariae*.

Three considerations, however, favor the alternative hypothesis, namely, that the host invasion has occurred from monkeys to humans:

(i) Humans are biologically (evolutionarily) more closely related to Old World monkeys (catharrhines) than to New World monkeys (platyrrhines). If lateral host switch from humans to monkeys were likely, it would be more likely that transfer would have been, first and most often, to our closer, rather than to our more remote relatives. This argument would be much weakened if the chimpanzee/gorilla parasite *Plasmodium rodhaini*, which is thought to be quite similar to *P. vivax*, or *Plasmodium schwetzi*, also a chimpanzee/gorilla parasite, which is similar to *P. malariae* [40], were shown to be genetically identical (or very similar) to the corresponding human parasites, so that they might have been recently acquired by the apes from humans. We note that, according to Gysin [40], "it has been shown" that *P. schwetzi* is "closely related" to *P. malariae*, but is "homologous" to *P. vivax*. This is a strange claim considering the considerable phylogenetic distance between *P. malariae* and *P. vivax* (see Fig. 11.2 and Table 11.2). We would point out, moreover, that *P. reichenowi* and *P. falciparum*, which are "closely related" and "homologous" [40], are evolutionarily as distant as their hosts, chimpanzees/gorillas and humans (see above). The catarrhine parasite *Plasmodium inui*, widely thought to be closely related to *P. malariae*, has been shown to be genetically quite different [31].

(ii) Humans and their ancestors have been geographically associated with catarrhine monkeys for millions of years, but only for several thousand years with platyrrhine monkeys. If the natural transfer from humans to monkeys were likely, it would have been much more likely that the transfer would have occurred to species with which humans have been in geographic association for a much longer period.

(iii) *P. simium* is parasitic to several platyrrhine species. A lateral host transfer from humans to monkeys would require several host switches, either from human to each monkey species, or from human to one monkey species and then from one to other monkey species, all in a short interval of time (a few thousand, or even a few hundred years). This state of affairs is more extreme when we consider the case of *P. malariae* and *P. brasilianum*; because this parasite's hosts include numerous platyrrhine species ([26] taxa are listed by [40, p. 420]).

Evolutionary parsimony favors the hypothesis that the host switch between *P. vivax* and *P. simium* and also between *P. malariae* and *P. brasilianum* may have been from primates to humans, so that *P. vivax* and *P. malariae* would have become human parasites only recently, perhaps only a few hundred years ago. The historical record (see above) is the strongest evidence against this conjecture. The matter can, in any case, be resolved by comparing the genetic diversity of the human and primate parasites. If the transfer from human to monkeys has been recent, the amount of genetic diversity in silent nucleotide sites (and other neutral polymorphisms) will be much greater in *P. vivax* than in *P. simium*, and in *P. malariae* than in *P. brasilianum* (combining in each comparison the polymorphisms present in the several monkey host species). A transfer from monkey to humans would be evinced by much lesser polymorphism in the human than in the monkey parasites.

The genetic indistinguishability between *P. vivax* and *P. simium* has recently been confirmed by an investigation of 13 microsatellite DNA loci and 8 tandem-repeat (TR) loci, which includes 108 *P. vivax* individual samples broadly representative of the distribution of this parasite [50]. Microsatellite polymorphisms arise at high rates by replication slippage, yielding new alleles with different numbers of the repeating unit. The genetic near-identity between *P. vivax* and *P. simium* is evinced, first, by the fact that all 13 microsatellite and 8 TR loci of *P. vivax* could be amplified in *P. simium*. The number of *P. vivax* microsatellite loci that could

be amplified in any one of seven other Old World (catarrhyne) monkey parasites range from zero (*P. gonderi*) to 10 (*P. cynomolgi*). Moreover, *P. simium* carries the same allele as *P. vivax* at the nine loci that are monomorphic in this species; the most common *P. vivax* allele at the three slightly polymorphic loci; and one of the *P. vivax* alleles at the only locus that is substantially polymorphic in this species. A parallel situation obtains at the TR loci, which evolve also rapidly. First, all eight TR loci could be amplified in the *P. simium*, but only three in *P. cynomolgi* or *P. fieldi*, and none in any of the other five catarrhyne parasites. Second, *P. simium* alleles are identical to those of *P. vivax* at all loci but one, at which the *P. simium* TR presents one private allele. This, however, is also the case for several local populations of *P. vivax*, each of which displays at least one private allele at one TR locus. A neighbor-joining tree based on TR genetic distances between populations includes *P. simium* within the *P. vivax* polymorphism [50]. Recent evidence shows that at least two independent host transfers involving *P. vivax* and *P. simium* have occurred between humans and platyrrhine monkeys [53].

Lateral transmission of *Plasmodium* parasites from monkey to humans is known for several species, including *P. simium* [25], *P. brasilianum* [17], *P. cynomolgi* [33], *P. knowlesi* [12], and, perhaps, *Plasmodium simiovale* [69]. Transmission from humans to monkeys can be accomplished experimentally [12] and may also occur naturally [14]. Among avian and reptilian malaria parasites, host shifts have been a common occurrence [9,78].

As pointed out above, the direction of host transfer between *P. vivax* and *P. simium* can be settled genetically. If the transfer has occurred from platyrrhine to humans, *P. simium* is expected to have greater nucleotide polymorphism at neutral sites than *P. vivax*. Ascertaining this will require the investigation of numerous independent DNA samples of *P. simium*, which are not currently available. Moreover, the issue may not simply be resolved, because the genetic impoverishment that is known to characterize *P. vivax* may be due to one or more recent demographic bottlenecks or selective sweeps, which has been postulated as the likely explanation for this impoverishment [50,53,70].

11.4 POPULATION STRUCTURE OF *P. FALCIPARUM*

The most consequential of the human malaria parasites is *P. falciparum*. As shown earlier, the lineage of *P. falciparum* has been associated with the human lineage during the evolution of the hominids, that is, since the separation of the human and chimpanzee lineages, about 8 million years ago. The question we now raise is whether the population dynamics of *P. falciparum* has or not followed that of its hominid hosts. A possibility, for example, is that *P. falciparum* may have been restricted through much of hominid and human history to some small locality from which it might have spread throughout other human populations as a consequence of increased virulence, environmental and demographic changes, or some other factors.

One way to approach this question is to investigate the distribution of genetic polymorphisms in *P. falciparum* populations. Numerous epidemiological studies have indicated that populations of *P. falciparum* are remarkably variable. Extensive genetic polymorphisms have been identified with respect to antigenic determinants, drug resistance, allozymes, and chromosome sizes (e.g. [7,21,46,49,60,81]).

Antigenic and drug resistance polymorphisms respond to natural selection, which is most effective in large populations – millions of humans are infected by *P. falciparum* and one single patient may harbor 10^{10} parasites [60]. The replacement of one allele by another, or the rise of polymorphism with two or more alleles at high frequency, may occur even in one single parasite generation. If the selection pressure is strong enough, all individuals exposed to the selective agent may die, except those carrying a resistant mutation. With populations as large as those of *P. falciparum*, any particular mutation is expected to arise in any one generation; and the same mutation may arise – and rise to large frequency – independently in separate populations. On the contrary, silent (i.e., synonymous) nucleotide polymorphisms are often adaptively neutral (or very nearly so) and not directly subject to natural selection. Thus, silent nucleotide polymorphisms reflect the mutation rate and the time elapsed since their divergence from a common ancestor. The population structure of *P. falciparum* is, consequently, best investigated by examining the incidence of synonymous polymorphisms.

The coalescence theory of population genetics assumes that the allele sequences of any given gene present in populations of an organism can be genealogically traced back to a single ancestral sequence, which is the most recent ancestor or "cenancestor" of all currently existing sequences (Fig. 11.3). If one ignores the possibility of multiple mutational hits (which is reasonable, so long as the sequences are not extremely polymorphic), the number of neutral polymorphisms observed in a sample of multiple strains will be a function of the neutral mutation rate, the time elapsed, and the number of lineages examined (and follow a Poisson distribution). If the neutral mutation rate can be established, the time elapsed since the cenancestor is simply determined by dividing the number of polymorphisms observed by the

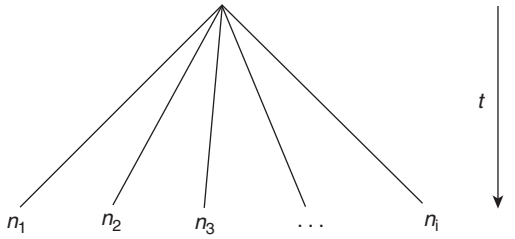

Fig. 11.3. Schematic representation of a star phylogeny showing the evolution of DNA sequences with the most recent common ancestral sequence (MRCA) at the apex. The scale of t represents the time elapsed since this MRCA gave rise to all its descendents in the extant population (n_1, n_2, n_3, ⋯, n_i).

TABLE 11.4. Single-Nucleotide Polymorphisms in 10 Protein-Coding Genes of Plasmodium Falciparum

Gene	Chromosome Location	Length (bp)	Sample Size	Polymorphic Sites		Number of Synonymous Sites	
				Nonsynonymous	Synonymous	4-fold	2-fold
Csp1 5′end	3	387	25	7	0	688	2,010
Csp1 3′end	3	378	25	17	0	1,050	1,625
Dhfr	4	609	32	4	0	2,144	4,128
Ts	4	1215	10	0	0	1,250	2,640
Mdr1	5	4758	3	1	0	1,350	2,088
Hsp86	7	2241	2	0	0	532	910
Dhps	8	1269	12	5	0	1,536	2,724
Calm	14	441	7	0	0	364	602
G6pd	14	2205	3	9	0	726	1,404
Rap1	—	2349	9	8	0	1,092	1,668
Tpi	—	597	2	0	0	180	262
Total	—	—	—	51	0	10,912	20,061

Source: Modified from Ref. 77.

mutation rate times the number of neutral nucleotide sites observed in the full sample [6,77].

Table 11.4 summarizes the polymorphisms found in a sample of 10 genes for which several sequences were available in DNA data banks [71]. The gene sequences analyzed derive form isolates of *P. falciparum* representative of the global malaria endemic regions. The *Dhfr* and *Ts* genes are found directly adjacent to one another on the parasite's fourth chromosome and encode the bifunctional dihydrofolate reductase-thymidylate synthetase (DHFR-TS) domain. Certain mutations in the *Dhfr* gene have been widely associated with *P. falciparum* resistance to anti-folate drugs, including pyrimethamine. Two other genes in Table 11.4 have been implicated with drug-resistant phenotypes of *P. falciparum*: the gene coding for dihydropteroate synthetase (*Dhps*) and the gene for multidrug resistance (*Mdr1*). The circumsporozoite protein (encoded by *Csp1*) is antigenic, and the rhoptry-associated protein (encoded by *Rap1*) may also be immunogenic. The other four genes in Table 11.4 are not known to be immunogenic or associated with resistance to any antimalarial drug currently in use. They code for calmodulin (*Calm*), glucose-6-phosphate dehydrogenase (*G6pd*), heat-shock protein 86 (*Hsp86*), and triose phosphate isomerase (*Tpi*). Six of the 10 loci exhibit amino acid polymorphisms, including the drug-resistance genes *Dhfr*, *Dhps*, and *Mdr-1*, as well as the antigenic *Cps* and *Rap1*. The significant result is that no silent polymorphisms are observed in any of the 10 genes.

11.5 MALARIA'S EVE HYPOTHESIS

Estimating time of divergence from neutral polymorphism requires that the neutral mutation rate (of third-position nucleotides in synonymous codons) be known. The neutral mutation rate has been estimated by comparing the *P. falciparum* gene sequences with *P. reichenowi* (the chimpanzee parasite), and also with a set of rodent *Plasmodium* parasites [77]. The number of neutral polymorphisms in Table 11.4 is zero, and, thus, at face value, the time elapsed since the cenancestor (t) would be zero, although it would become positive as soon as some neutral polymorphisms are observed. In any case, t is expected to follow a Poisson distribution, which allows calculation of the upper confidence limit for the time since the cenancestor. As shown in Table 11.5, the 95% upper confidence level is between 25,000 and 57,000 years ago, depending on which mutation rate estimate is used; the 50% upper confidence limit is between 6000 and 13,000 years. Rich et al. [77] have referred to this conclusion, that the world expansion of *P. falciparum* is recent, as the Malaria's Eve hypothesis.

In the few years since the Malaria's Eve hypothesis was proposed [77], the issue has been subject to a contentious debate. The initial conclusion of Rich et al. [77] was based on sequences that were then available from GenBank (see Table 11.4). The only criteria for inclusion of genes in the dataset was that they had to be void of repetitive DNA sequences and show no evidence of being under positive selection impacting synonymous codon substitutions. In 1998, the amount of sequence data available for the species was rather limited, but since that time the dataset has grown enormously, including the complete genome sequence of *P. falciparum* published in 2002 [36].

One of the new studies entails a large-scale sequencing survey of 25 introns, located on the second chromosome, from eight *P. falciparum* isolates collected among global sites [87]. The findings of this study confirm the previous result that there is an extreme scarcity of silent-site polymorphism among extant populations of *P. falciparum*. Among some 32,000 nucleotide sites examined, Volkman et al. [87] found only three silent

TABLE 11.5. Estimated Upper-Boundary Times (t_{95} and t_{50}, in years) to the Cenancestor of the World Populations of *P. Falciparum*. Mutation Rates are Estimated Based on Two Sets of Assumptions, Concerning Either the Origin of the *Plasmodium* Genus or the Time of Divergence Between *P. Falciparum* and *P. Reichenowi*. The *P. Falciparum* Cenancestor Lived More Recently than 24,511 or 57,481 Years Ago, with a 95% Probability; and More Recently than 5,670 or 13,296 Years with a Probability of 50%.

	Mutation rate $\times 10^{-9}$			
Assumption	4-fold	2-fold	t_{95}	t_{50}
Plasmodium radiation				
55 My	7.12	2.22	24,511	5,670
129 My	3.03	0.95	57,481	13,296
P. falciparum-P. reichenowi				
5 My	3.78	1.86	38,136	8,821
7 My	2.70	1.33	53,363	12,342

single-nucleotide polymorphisms (SNPs). Combining their data with the results summarized in Table 11.4, these authors estimated that the age of Malaria's Eve was somewhere between 3200 and 7700 years, depending on the calibration of the molecular clock.

Conway et al. [19] have presented further evidence in support of the Malaria's Eve hypothesis, based on analysis of the *P. falciparum* mitochondrial genome. They examined the entire mitochondrial DNA (*mtDNA*) sequence of four *P. falciparum* isolates originating from Africa, Brazil, and two from Thailand, as well as the chimpanzee parasite, *P. reichenowi*. Alignment of the four complete mtDNA sequences (5965 bp) showed that 139 sites contain fixed differences between *P. falciparum* and *P. reichenowi*, whereas only four sites are polymorphic within *P. falciparum*. The corresponding estimates of divergence (K, between *P. reichenowi* and *P. falciparum*) and diversity (π, within *P. falciparum* strains) are 0.1201 and 0.0004, respectively; that is, divergence in *mtDNA* sequence between the two species is 300-fold greater than the diversity within the global *P. falciparum* population. If we use the *rDNA*-derived estimate of 8 million years as divergence time between *P. falciparum* and *P. reichenowi*, then the estimated origin of the *P. falciparum* mtDNA lineages is 26,667 years (i.e., 8 million/300), which corresponds quite well with the earlier estimate based on the 10 nuclear genes shown in Table 11.4 [77]. A subsequent survey of *mtDNA* from104 isolates from Africa ($n = 73$), Southeast Asia ($n = 11$), and South America ($n = 20$), led to the conclusion that the extant global population of *P. falciparum* is derived from three mitochondrial lineages that started in Africa and migrated subsequently (and independently) to South America and Southeast Asia. Each mitochondrial lineage is identified by a unique arrangement of four polymorphic *mtDNA* nucleotide sites [18,19].

More recently, the complete 6-kb mitochondrial genome has been sequenced from 100 geographically representative isolates [48]. The sequences support a rapid expansion of *P. falciparum* in Africa, starting approximately 10,000 years ago. The data indicate, however, that some lineages are 5–10 times more ancient and that *P. falciparum* populations have existed in Southeast Asia and South America perhaps earlier than 50,000 years. This is unexpected, because if *P. falciparum* originated in Africa, the oldest polymorphisms should occur in that continent, as is the case for human mitochondrial lineages. Moreover, the presence of *P. falciparum* in South America more than 50,000 years ago is most unlikely, as humans colonized America 15,000–18,000 years ago (or, less likely, around 30,000 years ago) [11]. Moreover, it is generally accepted that *P. falciparum* malaria was introduced in America by the slave trade [88]. It seems likely that the high differentiation of some mitochondrial sequences may be a consequence of the large variation expected in a sample of non-recombinant neutral sequences, as is the case for the mitochondrial genome, or a consequence of natural selection increasing the frequency of some favorable sequences, which then would appear to be much older than they actually are. Some mitochondrial genes are likely to be under selection, such as the mitochondrial gene encoding cytochrome b, which seems to underlie susceptibility to some antibiotics [85].

A recent investigation of 20 protein-coding genes [42] has confirmed the low level of synonymous polymorphisms found in earlier studies of coding genes (Table 11.4; see also [31]). Table 11.6 presents the results. Many of the 20 loci were chosen so as to be different from those previously analyzed, and are located on 11 different chromosomes. The 20 genes were each sequenced in five to seven strains. Among the 22,611 nucleotides sequenced for each strain, there were 21 non-synonymous polymorphisms, but only one synonymous polymorphism, strongly supporting the Malaria's Eve hypothesis.

Arguments against the Malaria's Eve hypothesis come in two forms. The first argument is that the loci chosen in the studies supporting the recent world expansion of *P. falciparum* are a biased sample and do not reflect the levels of polymorphism in the genome as a whole. The second argument concedes that nucleotide polymorphisms are scarce and proposes that this is not attributable to recent origins, but rather reflects strong selection pressure against the occurrence of synonymous substitutions.

Some studies have estimated that antigenic polymorphisms in *P. falciparum* may be 40 or more million years old,

TABLE 11.6. Single-Nucleotide Polymorphisms in 20 Protein-Coding Genes of *P. falciparum.*

Gene Product	Chromosome	Nucleotide Per Strain	Single Size	Polymorphic Sites	
				nonsynonymous	synonymous
GPSP	1	552	6	0	0
AcCoA 695	2	2,667	7	11	0
AcCoA 685	2	2,706	5	2	0
ATPase 3	5	546	6	0	0
GPAT	6	636	6	0	0
STARP	7	1,092	6	1	1
GPRK	7	1,395	6	1	0
GPCA	9	252	6	0	0
GRP78	9	426	6	0	0
LSA1	10	684	6	2	0
GBP130	10	96	6	0	0
Enolase	10	738	6	0	0
Falcipain 3	11	1,350	6	0	0
FuFo1	11	1,890	6	1	0
CPN60	12	42	6	0	0
GPCE	13	1,725	6	0	0
Actin 2	13	1,131	6	0	0
G6PD	14	1,884	6	3	0
Falcipain 1	14	1,710	5	0	0
Aldolase	14	1,089	7	0	0
Total		22,611		21	1

Source: Modified from Ref. 42.

older than the origin of the hominids [45,46]. An analysis of nucleotide sequences for 23 gene loci has concluded that the cenancestor of extant populations of *P. falciparum* must be 300,000–400,000 years old [47]. As we have previously explained, these inferences ignore natural selection promoting rapid evolution of antigenic determinants; rely on erroneous sequence alignments that fail to recognize the presence of repetitive sequences; cannot account for large excess of non-synonymous over synonymous substitutions; and depend on poorly scrutinized sequences obtained from data banks [72–74]. Some alleged polymorphisms are sequencing errors because they come from a single gene derived from a single clone, but sequenced in different laboratories (see [42,74]). Similar problems plague the survey by Mu et al. [65] of more than 200 kb from the complete chromosome 3 and their conclusion that *P. falciparum* has maintained large populations for at least 300,000 years [42,74].

The argument that selection pressure against synonymous substitutions, rather than a recent cenancestor, can account for the scarcity of synonymous polymorphisms has also been answered in detail [71,74]. This argument is largely based on the predominance of AT pairs over GC pairs in the genome of *P. falciparum*. Indeed, the AT content of *P. falciparum* is 71.7% overall, and 83.6% in the third position [66]. It may suffice here to say that (1) AT excess lowers the rate of synonymous substitution, but does not altogether eliminate it; (2) in four-fold redundant codons, the bias favors codons ending in A or T but it does impact A↔T mutations; (3) other *Plasmodium* species are also AT rich and should have the same mutational constraints as *P. falciparum*, yet they exhibit abundant synonymous intraspecific polymorphisms as well as interspecific differentiation; (4) comparisons between *P. falciparum* and *P. reichenowi* at five genes for which data are available in both species yield high numbers of synonymous substitution (average $K_s = 0.072$ vs. $K_n = 0.046$ for non-synonymous substitutions) showing the AT richness does not impede synonymous substitutions [71].

11.6 THE NEOLITHIC REVOLUTION, AGRICULTURE, AND CLIMATE CHANGE

Beyond genetic inference, other considerations support the Malaria's Eve hypothesis. Sherman [80] has noted the late introduction and low incidence of *P. falciparum* malaria in the Mediterranean region. As pointed out earlier, Hippocrates describes quartan and tertian fevers, but there is no mention of severe malignant tertian fevers, which suggests that *P. falciparum* infections did not yet occur in classical Greece and the Mediterranean region as recently as 2400 years ago.

The expansion of *P. falciparum* across the globe after the Neolithic revolution, perhaps about 5000–6000 years ago, starting from a highly restricted geographic location, probably in tropical Africa, may have been made possible by (1)

changes in human societies; (2) genetic changes in the host–parasite–vector association that have altered their compatibility; and (3) climatic changes that entailed demographic changes (migration, population density, etc.) in the human host, the mosquito vectors, and/or the parasite.

Several authors have pointed out the significant consequences of changes in human living patterns, particularly the development of agricultural societies and urban centers that increased human population density [15,16,23,24,26, p. 237], and [54,80,90]. Genetic changes that have increased the affinity within the parasite–vector–host system may also have contributed to the recent expansion [4]. Coluzzi [15,16] has cogently argued that the recent worldwide distribution of *P. falciparum* has come about, in part, as a consequence of a recent dramatic rise in vectorial capacity due to repeated speciation events in Africa of the most anthropophilic members of the species complexes of the *Anopheles gambiae* and *A. funestus* mosquito vectors (see also [4]). Biological processes implied by this account ([2], see above) may have been associated with, and even been dependent on, the onset of agricultural societies in Africa ([1], see above) and climatic changes ([3], see above), specifically gradual increase in ambient temperatures after the Würm glaciation, so that climatic conditions about 6000 years ago in the Mediterranean region and the Middle East made possible the spread of *P. falciparum* and its vectors beyond tropical Africa [15,16,24,25]. Once demographic and climatic conditions became suitable for propagation of *P. falciparum*, natural selection would have facilitated evolution of *Anopheles* species that were highly anthropophilic and effective *P. falciparum* vectors [4,15,16,24].

The Malaria's Eve hypothesis is consistent with the evolutionary history of genetically determined immunity factors that confer resistance to *P. falciparum*. A glucose-6-phosphate dehydrogenase (*G6pd*) genetic deficiency occurs at high frequency in areas of malaria endemicity, particularly in Sub-Saharan Africa. Tishkoff et al. [83] have concluded that the *G6pd* mutants are only 3330 years old (95% confidence interval, 1600–6640 years), indicating that *P. falciparum* has only recently become a disease burden to humans. It has been known for decades that sickle-cell hemoglobin S (Hbs; βGglu→Val) heterozygosity confers protection against severe malaria [1,43]. Current evidence suggests that the sickle-cell mutation has arisen at least twice, once in India or the Middle East and once in Africa, although it is possible that the mutation may have arisen more than once in Africa [43]. Analyses of the β-globin gene haplotypes associated with a sickle-cell mutation in Africa suggest that it is recent in origin, perhaps no more than 2000 years [22,89]. A hemoglobin C (HbC; β6Glu→Lys) mutation seems to have arisen in Africa equally recently, or even more recently than hemoglobin S. HbC is associated with a 29% reduction in risk of clinical malaria in heterozygotes (HbCA) and of 93% reduction in HbCC homozygotes, which exhibit limited pathology compared to the severely disadvantaged HbSS ([64]; see also [34]).

Do we know whence in Africa *P. falciparum* emerged and dispersed throughout the world? A likely conjecture has been put forward by René Dubos [26, pp. 187–189]. Living species are rarely completely wiped out by epidemics, however virulent the parasite, because on the long-term host–parasite evolutionary dynamics yield some sort of equilibrium that permits the survival of both [32,51]. Over 90% of the Bush Negroes who live in the former Dutch Guinea become infected with *P. falciparum* shortly after birth, but, in contrast to what happens elsewhere in Sub-Saharan Africa, the infection causes little mortality among them. Nearly all adults harbor the parasite in their blood, but the intensity of parasitemia declines with advancing age. Splenomegaly decreases from more than 80% in infants to less than 15% in adults. The contrast with other African Negroes suggests, according to Dubos [26, p. 189], that the Bush Negroes have been in contact with *P. falciparum* for a much longer span of time. Only after the advent of slash-and-burn agriculture, and the evolution of effective anthropophilic *Anopheles* vectors, would *P. falciparum* have spread to other regions of Africa and elsewhere.

11.7 CONCLUDING REMARKS

The studies reported earlier in the chapter concern the population genetics and evolutionary relationships of *Plasmodium* parasites. In addition to their scientific import, these investigations are of great interest for public health. As pointed out earlier, malaria's toll in human lives and human suffering is enormous: yearly more than 11 million infant deaths and some 500 million adult clinical cases, as pointed out earlier. The economic consequences are also staggering. According to the World Bank, malaria slows economic growth in African countries by 1.3% per year, with an economic cost of about $3 billion per year and $100 billion over 30 years [59].

The Director General of the World Health Organization (WHO) announced in 1998 the Roll Back Malaria (RBM) campaign led by the United Nations with a goal of halving malaria deaths by the year 2010. The accomplishments are grossly inconsistent with that goal. According to WHO's own statistics, by 2004 malarial deaths have increased by nearly 20%, rather than decreased by 30% as anticipated by RBM [3]. WHO has estimated that in 2002 the total investment available for malaria control worldwide was approximately $200 million [91]. Yet, according to RBM, Africa needs $1 billion annually for malaria control. The United States and other rich countries have promised increasing investments in malaria control. In mid-2004, the Global Fund to Fight AIDS, Tuberculosis, and Malaria pledged $895 million over 2 years. This falls short of RBM's perceived needs, but it is a considerable improvement over current investments, if it indeed comes to pass. Another promising development comes from new donors, such as the Bill & Melinda Gates Foundation, which is injecting hundreds of millions of US dollars in malaria research.

The focus on economic investment, important as it is, often distracts from an essential component of control efforts: the human resources needed for implementing control measures in Africa and other developing regions [39]. The full benefits of scientific research accomplishments and increased investment in control measures cannot be achieved without well-trained and well-paid workers, who are distressfully in short supply in countries where malignant malaria is endemic.

One can only hope that increased awareness in rich countries of malaria's toll will yield the economic commitments and personnel investments that might one day, within less than one human generation, lead to the total (or near) eradication of malaria throughout the world, as smallpox, measles, and other scourges have been eradicated, or nearly so. Few if any other investments could do as much for humankind's welfare.

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GLOSSARY

Apical complex: A cell organelle characteristic of all protozoa of the phylum Apicomplexa.

Apicomplexa: A large group of protozoa, characterized by the presence of an apical complex at some point in their life cycle.

Clade: A group of biological taxa or species that share features inherited from a common ancestor.

Digenetic parasite: A parasite that passes through two different kinds of hosts in order to complete its life cycle; it reproduces sexually in one of the hosts, asexually in the other.

DNA amplification: Multiplication of copies of a DNA sequence that allows analyzing the sequence.

Genetic distance: A measure of genetic differentiation between homologous (sharing a common ancestor) genes from different organisms; or of genetic differentiation between organisms.

Host transfer and/or host switch: A "horizontal" shift of a parasite from one host species to another, distantly related host species.

Monogenetic parasite: A parasite that lives throughout its entire life cycle on a single kind of host.

Polymerase chair reaction (*PCR*): A molecular biological technique for amplifying (creating multiple copies of) DNA without using a living organism.

Phylum: The major taxonomic category of animals, plants and other organisms; contains classes.

Sporogony: Reproduction by spores; in *Plasmodium*, formation of spores typically containing sporozoites that results from the encystment and subsequent division of a zygote.

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CHAPTER 12

Ecology Of Infectious Diseases: An Example with Two Vaccine-Preventable Infectious Diseases

H. Broutin,[1] N. Mantilla-Beniers,[2] and P. Rohani[3]

[1]*Unit of Research 165 "Genetics and Evolution of Infectious Diseases," UMR CNRS/IRD 2724, Institute of Research for the Development (IRD), BP 64501 34394 Montpellier Cedex 5, France*

[2]*Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, United Kingdom and Instituto Gulbenkian de Ciência, Apartado 14, 2781-901 Oeiras, Portuga*

[3]*Institute of Ecology, University of Georgia, Athens, GA 30602, USA*

12.1 INTRODUCTION

It nowadays often comes as a surprise to the non-specialist that ecology and infectious diseases can be integrated in an area of study. The irony is that they have a common origin and only came to exist as separate fields with scientific development and the advent of reductionism.

The dissociation of natural science into subfields such as immunology, pathology, molecular biology, and genetics has led to key scientific breakthroughs, some of which make up the foundations of modern infectious disease epidemiology. In particular, scientific evidence of the mechanism of contagion presented epidemiologists with fundamental answers and led to reformulating central questions in the field. Another consequence of our improved understanding of disease transmission is that mathematics began to be used as an explanatory [27] and, more recently, predictive tool [2,17,31] in the study of infectious disease epidemiology.

Interestingly, the host–parasite interactions captured in epidemic and demographic data are part of the study matter of population ecologists. As a result (cf. Section 12.1), ecology has lent useful concepts to epidemiology, and finds in the latter a fruitful test-bed for its theoretical developments. Moreover, changes in social habits (e.g., increasing frequency and range of travel), demography, habitat structure (e.g., urbanization) and the environment (e.g., deforestation, temperature) are responsible for recent, unexpected increase in the importance of infectious diseases as causes of mortality in an increasingly complex ecosystem [12,34]. In this context, humans cannot be considered as "particular case" or a "particular host" for pathogens. Emerging or re-emerging diseases affect many animal and plant species and, as such, humans should not be considered as a special host; from an evolutionary perspective, humans might even be a "bad" host in many cases.

In fact, the study of human infections must be linked with "ecosystem health" putting in light the *strong relation between biodiversity and human health* [5,41]. Thus, in order to face up to the challenge presently posed to epidemiology, it will be necessary to reinforce its links with ecology, and also seek to understand how pathogens evolve.

The ecological study of infectious diseases [22] places the individual within a population in a given environment, thereby analyzing the spread of diseases over different scales of time and space. Its goal is a global understanding of the development (emergence, spread, etc.) and persistence of a disease in a host population that integrates the biology of the host–pathogen association (pathogen life history, host demography, social habits, etc.) and the influence of environmental factors (e.g., precipitation, temperature).

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

The present wealth of scientific knowledge might make it impossible for modern scientists to apprehend all what once was the realm of natural historians, but interdisciplinary collaboration can (and should!) be used to bring together areas of knowledge that relate to present problems.

In this chapter, we will first present some of the main concepts and methods currently used in the study of infectious diseases. In the second and last section, we will illustrate how these tools are implemented using results obtained by our groups in the study of pertussis and measles epidemiology.

12.2 CONCEPTS AND METHODS

12.2.1 Mathematics—Modeling

The first use of mathematics in epidemiology is generally attributed to the Englishman John Graunt, who in the seventeenth century proposed that greatest progress would be made in the battle to understand the causes of human mortality by quantifying their rate through time. Although this effort was largely *descriptive* [19], it was of paramount importance because it laid the basis for quantitative reasoning and the collation and comparative analysis of public health data. Modern techniques of time series analysis and the development of computers have made it possible to capture essential information that was formerly lost in statistical studies [10,20].

With the development of germ theory came the first mathematical models that incorporated assumptions on the mechanism of contagion. The mechanistic approach was used to try to *explain* patterns found in epidemic data [27]. It has produced fundamental theoretical progress and remains highly productive.

The initial attempts to *predict* epidemic spread using extant data were hindered by the lack of detailed epidemiological and demographic information, the computing demands of the models and gaps in the knowledge of disease transmission. However, recent ventures have concluded with success [2,17,31], highlighting the role that mathematics can play in modern epidemiology. One of its main applications is the prospective study of alternative control strategies.

More details are given in Chapter 23 (M. Choisy, J.F. Guégan, P. Rohani)

12.2.2 Population Ecology

As mentioned in Section 12.1, the link between population ecology and epidemiology is rather natural, because the former seeks to understand what causes temporal and spatial changes in population abundance and how these changes relate to environmental factors and to those intrinsic to ecological interactions – and the latter focuses on host–pathogen dynamics.

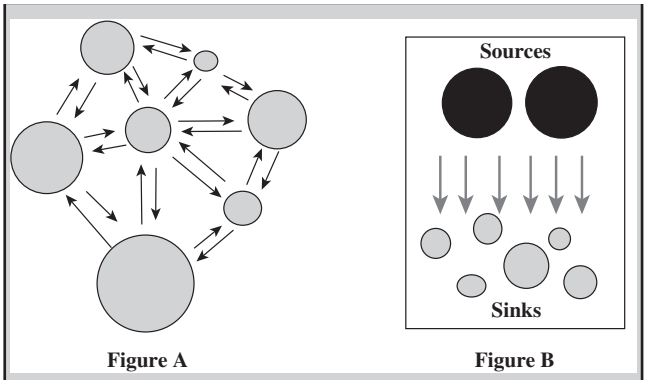
Population ecologists rarely have access to data of the spatio-temporal span and resolution that can characterize disease records. Furthermore, host demography, social habits, and environment are often well documented in parallel to pathogen dynamics, particularly in the case of human populations. As a result, important parameters of the two central populations are known. Exceptional data sets even contain information from various host and pathogen populations, thus providing the opportunity for comparative studies. Lastly, the impact of large-scale perturbations, such as sudden demographic changes or the start of mass-vaccination, is reflected in some data sets. These perturbations equate to environmental changes (e.g., habitat fragmentation) that would be questionable interventions in other ecological systems, therefore providing invaluable information to conservationists.

12.2.2.1 Persistence and spread of infectious diseases—metapopulation concept *Population ecology* is concerned with infectious dynamics at spatial and temporal scales that differ from those used in other disciplines studying infectious diseases. At the largest scale, it seeks to understand disease behavior in the population of an entire geographic region. The aim is to answer different basic questions that are relevant *because* they reflect what affects disease incidence: *Is the disease predictably periodic? Do epidemics occur everywhere at the same time? Can the disease persist over time, or does it go extinct? How does disease spread between geographically isolated host populations? Is disease transmission the same today as it was 50 years ago*?

Population ecology looks in particular at the spatial structure of communities and its consequences on the *persistence* and *geographic distribution* of species [42]. A concept from population ecology that is now highly developed for the study of infectious diseases is the concept of *metapopulation* [28,33]. A metapopulation is loosely defined as a population of subpopulations interconnected by immigration (see Box 12.1). For a

BOX 12.1: METAPOPULATION CONCEPT

A *metapopulation* (Fig. A) is defined in ecology as a population of subpopulations (gray circles) interconnected by immigration (black arrows) [28]. The dynamics of the entire metapopulation will depend on the *extinction and recolonisation* of its constituent subpopulations (or habitat patches). Metapopulation dynamics will depend mainly on (i) *local dynamics* (which at a fundamental level depend on the size of the subpopulation) and (ii) *population flux* between patches, with the proviso that migration rates are sufficiently low so as not to affect local dynamics *per se*. An interesting metapopulation configuration is the "*source–sink*" structure, where only one direction of population flux is relevant to local and global population dynamics (cf. Fig. B). In this case, the dynamics of big populations (called "sources," in black) shapes abundance patterns in small populations (called "sinks," in gray) and therefore defines metapopulation dynamics.



Applied to infectious diseases, habitat patches are human groups that can be defined at many different spatial scales, from, for example, families or neighborhoods (local scale) to countries or continents (global scale).

given species, we can thus study the global dynamics of the populations, taking into account the different community sizes of subpopulations and the flux of individuals between them.

Applied to the study of infectious diseases, subpopulations correspond to human communities that can be considered at different spatial scales (e.g., family, city, country). This approach has been applied to the study of measles and pertussis at a country level [23,37] and a very fine rural scale [9].

Moreover, in the study of disease persistence, the relation between population size and the duration of infection fade-outs (periods when the disease cannot be detected in the host population) can be used to estimate the infection's *Critical Community Size (CCS)* (see Box 12.2). The CCS [6,7] is defined as the population size below which the disease cannot persist. Vaccination strategies are expected to decrease disease transmission and increase the CCS.

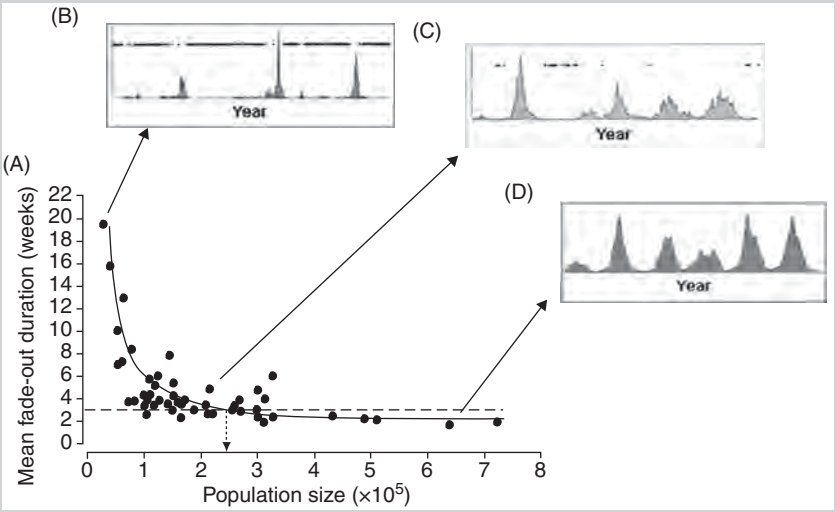
Another issue that can be studied within this framework is the detailed spatio-temporal dynamics of the pathogen

BOX 12.2: CRITICAL COMMUNITY SIZE (CCS)

One way to estimate the CCS for a given disease is to plot the mean duration of disease extinction (i.e., fade-out) in relation to population size. A fade-out is defined based on the average time that a patient takes to recover from infection counted from the moment in which transmission occurred. Only during this time can the patient him/herself transmit the infection. In the case of measles, for example, this is approximately 2 weeks. Therefore, when no new cases are reported in 3 weeks or more, it is safe to assume that the chain of transmission is broken and that the pathogen has gone extinct in that host population.

Let us illustrate the CCS with an example (see figure below) extracted from Rohani et al. [38], who studied pertussis times series in 60 cities in England and Wales during the vaccination era (1957–1974). Graph (A) represents the mean duration of fade-outs (in weeks) in relation with the population size of each locality. As you can see, the bigger the locality, the shorter the period of disease extinction. To determine the CCS, we focused on the threshold of 3 weeks for pertussis (if a new case is reported in the locality more than 3 weeks after the last one, it cannot be due to transmission within the locality and must be the result of an infectious contact with someone from outside the locality) represented by the blue dot line. Below this threshold, the disease persists and above it, goes extinct.

The CCS will correspond to the population size at the intersection between this threshold and the fade-out duration curve (see dot arrow), here around 250,000 inhabitants. For localities with a population size below this CCS, the disease goes extinct (see (B) and (C) as examples), whereas there is no extinction of the disease in the localities with a population size above CCS (see (D) as example).



metapopulation. One question is as follows: *Does the disease spread between all communities without any direction or does the disease spread in a special direction*? This question is important for the control of infectious diseases, because it can help to identify potential sources of infection. Here, the mainland–island paradigm of ecology [28] finds a counterpart in the "*city–village*" concept proposed by Anderson et al. [3]. They suggested that diseases spread from big cities to small villages, which corresponds to a particular metapopulation structure, namely the "source–sink" paradigm (see Box 1). Analyses of ecological time series have shown that measles and pertussis indeed spread following a size-hierarchy, both in England and Wales and in a small rural area of Senegal (*cf.* Section 12.3) [8,21,25].

12.2.2.2 Periodicity and synchrony of epidemics—times series analyses The temporal dynamics of infection are studied using various methods of *time series analyses* of disease cases. A time series is defined as a series of observations ordered in time, for instance, the monthly number of new cases for a given disease in a given host population. Different methods can be used to investigate the periodicity of epidemics and the study of the synchrony with which they occur in different subpopulations. In ecology, time series analyses have been used to determine the degree of *coherence* in oscillations in abundance of separate animal populations [29,30] and to study the *periodicity* of these fluctuations population in relation to geographical gradients [32]. A direct application of this approach concerns conservation biology [28]. Indeed, for a given species, *synchrony* of different populations in different geographical locations (i.e., populations in the same dynamical state simultaneously) implies an increase in the risk of extinction. In contrast, if population dynamics are not synchronized, then the extinction of one local population can be balanced by recolonization from a neighboring population. This is termed the *rescue effect* in ecology, and effectively enhances the overall chances of persistence of the species.

The same rationale can be applied to pathogen populations. Using infectious disease data of unique temporal span and spatial resolution, various studies [3,16,20,38] have characterized the periodicity and synchrony of measles and whooping cough epidemics before and after the start of mass vaccination. When epidemics are incoherent, disease extinction in one population is only temporary, because disease is later reintroduced through contact with infectious individuals from other populations.

Thus, this new approach to epidemiological issues is primordial for better understanding the patterns of disease spread in space and time, and naturally inspects issues that are relevant to disease control. In the second part of this chapter, we will illustrate this approach with studies of pertussis and measles dynamics at two different spatial scales and in different environments. To obtain an integrated picture of disease spread, it is crucial to compare different environmental and demographic conditions.

12.2.3 Comparative Approach—The Search for Emerging Themes?

Although major research developments have recently come about in other fields of life science, that is, population dynamics, community ecology, and macroecology [32], often through the use of a comparative research perspective, epidemiology continues to suffer from a lack of comparative studies.

With recent evidence of the impact of large-scale phenomena, for example, climatic change, on infectious disease patterns [34], the recognition of the importance of regional or even global processes interacting with microbe population dynamics in local human communities has become evident. Modern epidemiology is now confronted with the problem of how to identify the spatio-temporal and organizational scales that might be relevant in explaining disease patterns and processes. Many investigations on childhood diseases have provided clear evidence of how large-scale studies are of substantial interest for public health [4,21,38]. There is now a growing scientific tendency, under the impetus of population biologists, to provide a broader perspective on epidemiological systems in order that only the important disease generalities or patterns remain. This approach is called *comparative analysis*, and it consists of the comparison, on a broad spatial scale, of long-term data for a given disease across different localities. The main focus of comparative analysis in general is to contrast data acquired at a smaller spatial scale and to consider that emerging patterns may exist at a larger scale encompassing the total data set under study. The basic role of comparative analyses in epidemiology is to describe the different spatio-temporal patterns that may be at work on the different hierarchical scales under scrutiny, and then to explore the corresponding processes responsible for the observed patterns.

Comparative studies of pathogen population dynamics are thus a promising way to explore public health issues, offering a much broader perspective on health and a more quantitative approach with which similarities and specificities in the behavior of infectious diseases can be distinguished. Such studies, based on an ecological understanding of infectious diseases, should help us to improve and adapt the means for controlling these infections (using vaccination, for example) on a global scale.

12.3 AN EXAMPLE WITH TWO DIRECTLY TRANSMITTED DISEASES: MEASLES AND PERTUSSIS DYNAMICS

12.3.1 Pertussis and Measles: Two Vaccine Preventable Diseases

Pertussis and *measles* are two ubiquitous *vaccine-preventable diseases* of humans. Both are highly infectious and are transmitted in aerosol droplets following contact between infected and susceptible individuals. Pertussis (also called "whooping cough") is a respiratory disease caused by Gram-negative bacteria of the

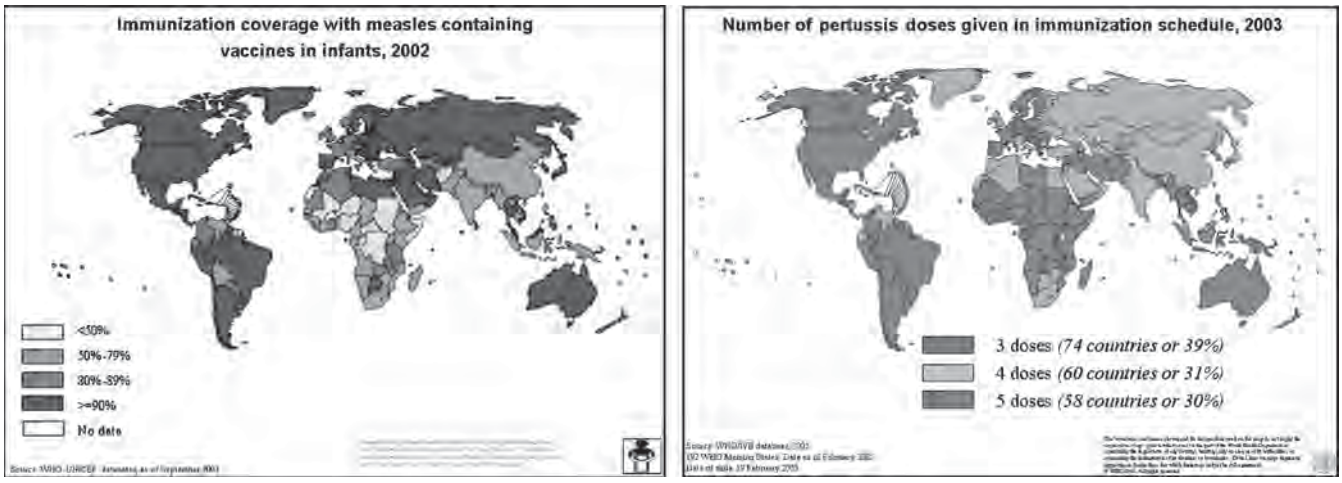


Fig. 12.1. World maps showing vaccination coverage against measles (left panel) and pertussis (right panel). These maps were extracted from the WHO website http://www.who.int.

species *Bordetella pertussis*. Individuals infected with pertussis become infectious after an incubation period of approximately 8 days, during which the bacterium spreads and proliferates in the host. They are then infectious for approximately 14–21 days. Measles is due to a paramyxovirus (see chapter 9 for classification). In this case, an incubation period of 8 days on average is followed by approximately 5 days during which patients remain infectious. Active immunity results from either recovery to natural infection, or vaccination.

In developed countries, large-scale vaccination programs against both infections started between 40 and 60 years ago. In many developing countries, however, systematic vaccination was initiated only recently (1974) via the Expanded Programme on Immunisation, which has been implemented in Africa since the mid-1980s (Fig. 12.1). Global incidence of both infections has been dramatically reduced as a result of vaccination, but *measles and pertussis remain an important public health problem in developing countries* [43,44]. Furthermore, *in several developed countries a resurgence in whooping cough has been detected in the last decades* [13,14], *despite high vaccine coverage* [15,26].

The unrelenting toll of life taken by these infections has motivated the collection of records of morbidity and mortality in numerous human populations around the globe. In particular, disease reporting has rendered detailed incidence reports that date back to the years preceding the start of vaccination campaigns from regions in two countries of disparate social, demographic, and economic conditions: the *Niakhar* in Senegal[1] [8,9].[c1] (Fig. 12.2), and *England and Wales* in the United Kingdom [37]. Each of these data sets is made up by weekly reports from geographically separate human populations that are linked epidemiologically by human travel.

Niakhar is a small rural area located around 150 km east of Dakar in Senegal. It is constituted by 30 localities with population sizes ranging from 50 to 3000 inhabitants (see Figs. 12.3 and 12.4). Pertussis and measles cases have been reported since 1983, and vaccination started at the end of 1986. Data for England and Wales originate from the largest 60 towns and cities in the area. Sizes range from 20,000 inhabitants in Teignmouth to over 3 million in London. British data span from 1944 to 1994, and mass vaccination started in 1957.

In what follows, we present studies of the spatio-temporal dynamics and persistence of pertussis in each area. These analyses draw parallels between the two regions, comparing the manner in which immunization programs altered disease dynamics in each case. Study methods have a strong base in ecological studies. We hope they make apparent *the fruitful interchange of ideas between ecology and epidemiology* that was outlined in the first part of this chapter.

12.3.2 Persistence—CCS and Impact of Vaccination

As detailed before (cf. Box 2), the CCS of an infection can be estimated by counting the number of weeks in which no cases are reported in a given host population and noting how the duration of disease fade-outs relates to population size. Figure 12.5 shows results obtained for pertussis in England and Wales [38] and in Niakhar, Senegal [9].

Visual inspection of these figures shows two important similarities between British and Senegalese data. The first of them concerns the general shape of the relation. In both cases, the larger the host population, the shorter the duration of its disease fade-outs. Indeed, the *disease persists better in large populations*, where the number of individuals susceptible to infection is large enough to maintain the chain of transmission. A second point that is evident from these figures is that fade-outs are longer after the start of immunisation campaigns (black vs. gray curves). Thus, *vaccination effectively reduced disease persistence in both England and Wales and in Niakhar.*

It is important to remark that the largest towns in Niakhar are roughly an order of magnitude smaller than the smallest

[1]The Institute of Research for the Development (IRD) performed the demographic (since 1963) and epidemiological (since 1983) survey in the Niakhar population, a small rural area in Senegal.

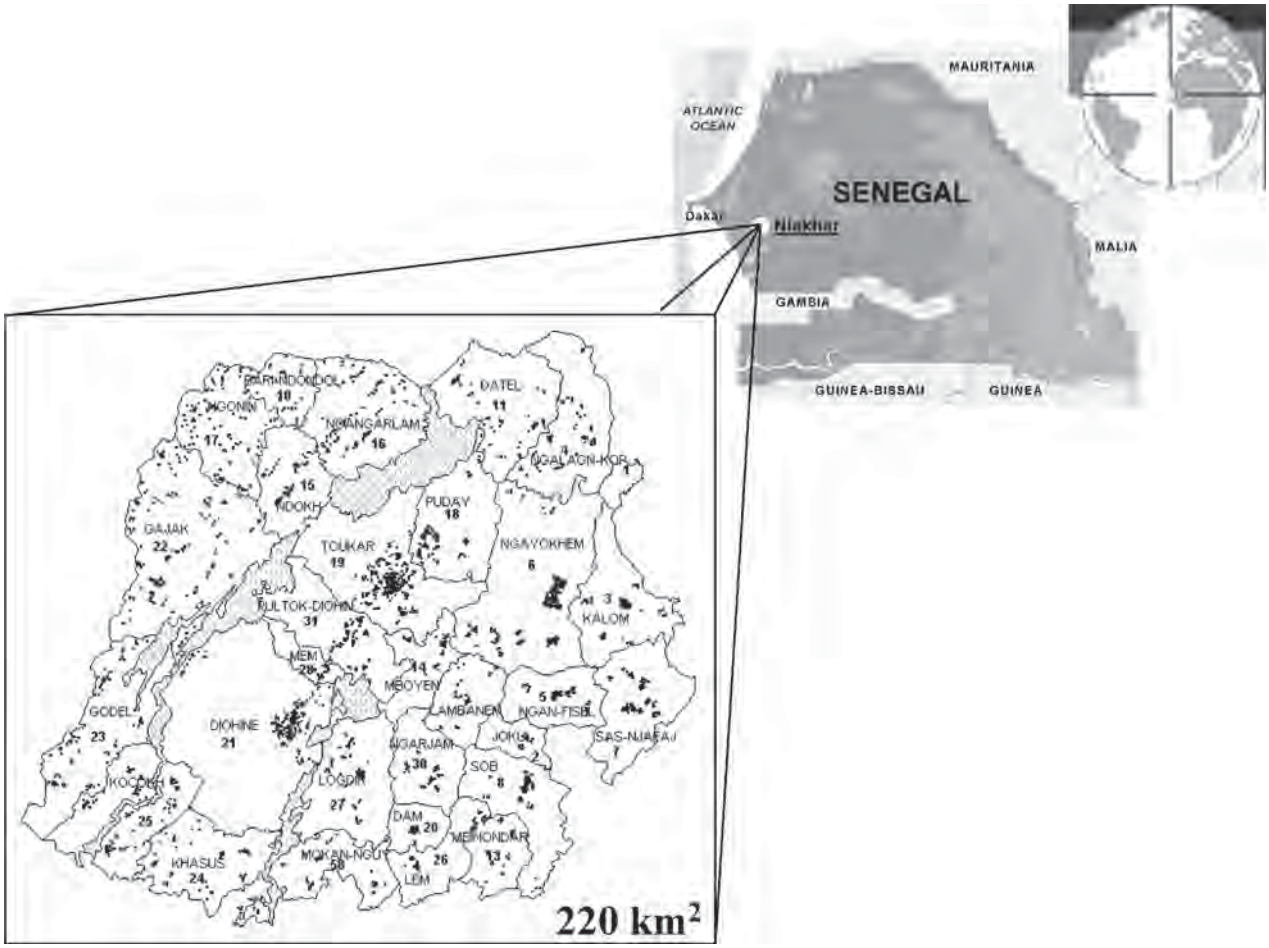


Fig. 12.2. Location and map of the Niakhar area in Senegal. Gray areas correspond to backwaters during the rainy season. Villages are delimited by the black lines. Black dots represent compounds – groups of houses – and thus exhibit the geographical distribution of human populations within Niakhar.

Fig. 12.3. Picture of the *Niakhar* area, Senegal, in dry season. The area is constituted by 30 villages. Each village is divided into hamlets, themselves composed of "compounds." The compound, representing the smallest structure of the zone, corresponds to a group of houses where extended families live, occupying one, or several, households (photo: H. Broutin).

Fig. 12.4. Picture of inhabitants of a big compound in Niakhar, Senegal (photo: H. Broutin).

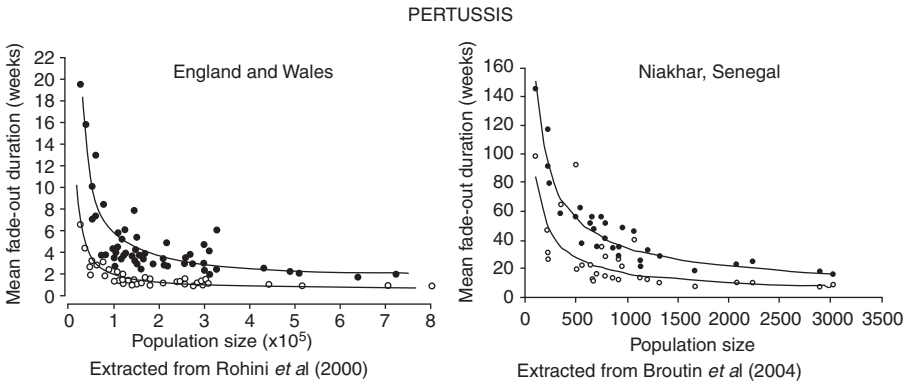


Fig. 12.5. Mean duration of fade-out (in weeks) in relation to community size for pertussis in England and Wales (left graph) before vaccination (1944–1957, in gray), and after vaccination (1957–1974, in black) and in Niakhar, Senegal (right graph), before vaccination, that is, 1983–1986 (in gray) and in vaccine era, that is, 1987–2000 (in black). It is important to notice differences in scales between the two graphs.

populations found in the British records analyzed here. Interestingly, fade-out duration in populations in Niakhar appears to extend the results obtained for England and Wales. The range of population sizes represented in British data includes places where the disease does not fade-out and so allows for a direct estimate of the CCS of pertussis before and after the start of immunization [38]. In contrast, the much smaller population of Niakhar means that there are periods in which pertussis is absent from the entire region (Fig. 12.6). Thus, its recurrence after a fade-out depends on contacts with infected individuals from outside Niakhar.

Thus, *the reduction in transmission brought about by vaccination can be observed at very different spatial scales and in communities of very different socioeconomic and demographic characteristics*.

Epidemiological information from all the communities in a geographical region (Niakhar) permitted an unprecedented study of the spatial spread of pertussis [8,9]. This is presented in the next section, along with an analogous study of measles dynamics in England and Wales [21].

12.3.3 "CITY–VILLAGE" SPREAD

Based on theoretical studies, Anderson et al. [3] first proposed that infections spread following a size hierarchy from cities to villages (the "city–village" paradigm). This was later confirmed by different empirical studies of measles cases in the British Isles and the United States [11,20,21], which showed that infection progressively diffuses from urban centers to the surrounding rural areas, and at regional and large scales. The phenomenon of infectious disease diffusion from big cities to smaller localities could be quite important in terms of infectious disease control. Indeed, the identification of "sources" of infection could be used to target vaccination efforts. For this reason, it is very important to study the spatio-temporal dynamics of infectious diseases in different contexts.

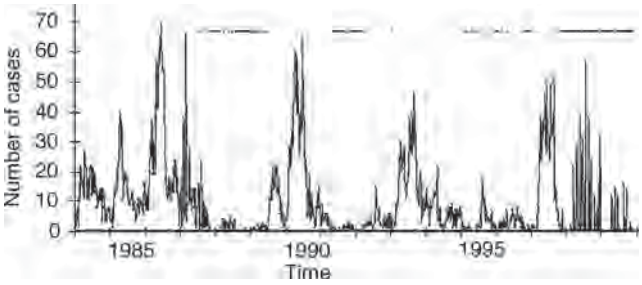


Fig. 12.6. Weekly cases of pertussis in all of Niakhar (Senegal, 1984–1999). Black dots at the top show weeks in which no cases were recorded.

Measles diffusion in England and Wales was analyzed using morbidity reports from 845 towns and cities and 457 rural districts [21]. In this analysis, only the 60 largest cities are classed as "urban" populations. The remaining time series are then aggregated in a "rural total." The proportion of total cases reported in each "urban" population was then compared to the proportion of total cases found in the rural total time series. Their relation was characterized by their Pearson correlation coefficient, and negative correlations correspond to populations in which epidemics take-off before they do in the rural total.

Correlation coefficients are plotted in relation to population size in Figure 12.3A. Correlation coefficients between the rural total and time series from large populations (as proportions of the total cases) are usually negative, showing that measles spreads from the largest cities (e.g., London or Birmingham, which correspond to the two largest green disks in Fig. 12.7) to the surrounding area. Populations above the CCS serve as "reservoirs" from which measles spreads to rural settlements.

Similar analyses were performed in the small area (220 km^2) of Niakhar in Senegal for pertussis [8] to test the idea that the "cities and villages" model might also be relevant on a finer spatial scale. As before, we defined a rural total, made up of all but the two largest cities in Niakhar (Toukar and Diohine). Our study suggested that disease progresses from the largest

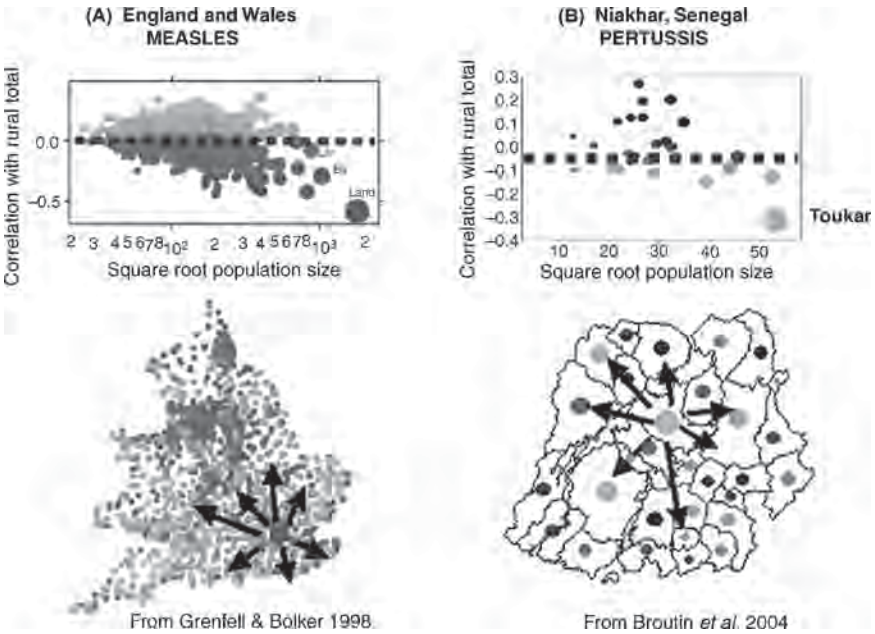


Fig. 12.7. Illustration of the comparison of infectious disease spread at two different spatial scales. The left part (A) describes the spread of measles in England and Wales (*cf.* [21]). The right part (B) shows pertussis spread in the rural area of Niakhar, in Senegal (cf. [8,9]). In the latter, orange (respectively, green) denotes negative correlations between measles in a population (as proportion of total cases) and measles in a "rural" aggregate (see text for details). For each part, the top graph provides on the *y*-axis the correlation between the "rural" aggregate and the time series in individual populations (845 localities in A and 30 in B), in relation with the population size (on the *x*-axis). A negative correlation describes a delay between rural cases and epidemic increase in the study population. The bottom maps are a spatial representation of the upper graphs. More analyses (not shown here) confirm that cases appeared first in the biggest localities and then in rural populations (black arrows symbolize disease diffusion). See color plates.

town (Toukar) to the rural surroundings (see Fig. 12.7B). Indeed, negative correlations between "urban" populations and the rural aggregate are indicative of an urban–rural hierarchy in pertussis epidemics in Niakhar after vaccination. Epidemics in "urban" populations (which have markets, bus stations, and health centers) begin and reach their peak between 10 and 15 weeks before "rural" epidemics, in conformity with results obtained for measles in England and Wales [21].

As noted before (Section 12.3.2), whooping cough cannot persist in Niakhar without external input of new cases (see Fig. 12.5 the CCS is not reached in Niakhar). We can now add a new fact to this mechanism: cases arrive initially in the biggest villages, Toukar, before spreading to the surrounding areas. Even though this pattern of disease spread can explain the spatio-temporal dynamics of pertussis in the studied area, it must be noted that other mechanisms (e.g., pertussis arriving directly in the small villages from an external source) cannot be ruled out. Thus, a size hierarchy could potentially determine the spatio-temporal dynamics of an infection even in effectively rural areas from which the infection fades out after epidemics.

This type of approach needs to be completed by other studies, because it could be helpful for adapting vaccination strategies. If spread of disease from urban centers to rural counties can be generalized, then this mechanism could imply new strategies for vaccinations, with less expanded but more precise programs that would be more realistic in the field, particularly in developing countries. This new type of research should lead to better control of disease using targeted vaccination.

12.4 CONCLUSION

The example in Section 12.3 illustrates how the interchange of ideas between ecologists and epidemiologists has contributed to our understanding of pathogen population dynamics. Some of the theoretical developments derived from this approach have in fact led to concrete suggestions aimed at improving vaccination strategies [1,35]. Other studies with a similar perspective have shown that ecological interactions *between* pathogens might also have consequences on disease dynamics [36,39]. This suggests that the interaction

between a pathogen and its host need not be the only one relevant to epidemiologists.

Definitively, *ecology and epidemiology need to develop stronger links, both with each other and with more, relevant, disciplines such immunology and microbiology*. Ecology is based on the population-level studies of ecosystem dynamics and interactions. Infectious diseases or pathogen populations suffer the same ecological and evolutionary laws experienced by other living beings. Whatever the name ("Medical ecology," "Eco-epidemiology," or "Ecology of health"), the coupling of both ecology and epidemiology is now the necessary way of research to better understand and control infectious diseases in this fast evolving word. In fact, much remains to be done to control preexisting infections, and emerging and re-emerging infections present novel challenges to epidemiologists. The magnitude of the challenge is enormous. It is therefore essential to maintain a close collaboration between epidemiologists and ecologists. Yet it is similarly important to expand interdisciplinary links and strengthen the relationship with, for example, population geneticists that can potentially lead to predicting pathogen evolution and emergence (18,24,40).

The task ahead will also require a coordinated effort to gather information at different biological levels of organization (from molecular scale to ecosystem). Indeed, the lack of reliable time series constitutes a major brake to the understanding of population dynamics in epidemiology. For that, standardized health surveys that reflect *pathogen dynamics* and keep track of *host habits* and *demography* will be indispensable. *Population genetics* will be a necessary complement. Additionally, *data on environmental changes* and their effect on species diversity and habitat composition will be needed to complete the picture. In fact, *we need first to observe and understand patterns in order to then discover the processes that shape them*. In the light of the challenges presented by the modern world, it is of fundamental importance to bring together our efforts to understand it.

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CHAPTER 13

Influenza Evolution

Robin M. Bush

Department of Ecology and Evolutionary Biology, University of California, Irvine, USA

13.1 INTRODUCTION

The great majority of influenza viruses are slowly evolving avirulent residents of the intestines of birds. On rare occasion, an avian influenza virus jumps the species barrier and infects a human or other animal. The results can be disastrous: approximately 40 million humans were killed by the 1918 "Spanish flu." Even the best public health care systems today are not prepared to deal with mortality on this scale [95].

We are currently witnessing what many people fear is the start of the next pandemic. Influenza A viruses of the H5N1 subtype began causing widespread outbreaks of disease in poultry in 1997 in Southeast Asia. Direct contact with infected birds has resulted in the deaths of at least 54 people. Prior to 1997, many people thought that avian influenza viruses could not directly infect humans. This is clearly not the case.

An understanding of influenza evolution is critical if we wish to cope with this potential crisis. For example, we know that we must anticipate the emergence of resistant strains if we try to stop a pandemic with antiviral drugs. Despite this knowledge, adamantadines, the most accessible class of anti-influenza drugs, have apparently been added to poultry drinking water in China for years. These drugs are now considered useless for pandemic control should the H5N1 strain begin to spread.

Lacking a H5N1 vaccine and adequate access to effective antiviral drugs, we are currently quite defenseless against this strain. Fortunately, H5N1 viruses have not yet evolved a way to effectively transmit between humans. We do not know the exact mechanisms that would allow such transmission, or how to prevent it from happening.

This is particularly humbling given that there have been major breakthroughs in recent years in many areas of influenza research. Past pandemic strains have been sequenced, including the 1918 "Spanish flu." Reverse genetics have been used to create influenza viruses *de novo*, allowing direct examination of the contributions of various genes and mutations to virulence and host receptor specificity. Yet when the question arises as to whether we are seeing the evolution of the next human influenza pandemic strain, the answer is that we simply do not know.

My goal for this chapter is to introduce the basics of influenza evolution. I first describe the influenza virus and its genome. I then discuss evolution within birds, the primary host of influenza. I briefly cover influenza in swine and horses, species that, like humans, carry a limited number of avian-derived influenza strains. I delve most deeply into the evolution of influenza in humans. I cover not only those topics that are necessary to understand potentially devastating evolutionary events such as pandemics but also those relating to the more familiar face of epidemic influenza – which we all know from being reinfected continually throughout our lifetimes. I end with a discussion of how evolution impacts influenza intervention, with particular application to the current H5N1 avian influenza epidemic in Southeast Asia.

13.2 THE INFLUENZA VIRUS

The influenza viruses comprise three genera in the family Orthomyxoviridae. The genera are commonly referred to as influenza "types" A, B, and C. Influenza A and B viruses cause winter epidemics in humans. Influenza C viruses cause sporadic local outbreaks of relatively mild respiratory disease in children.

Influenza A viruses infect a wide variety of birds, primarily waterfowl, shorebirds, and gulls. Avian influenza A viruses are thought to be the ancestors of influenza A strains currently circulating in swine, horses, and humans. The origins of

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

influenza types B and C, which infect only humans, are unknown. Types B and C are clearly related to type A viruses. They may be the descendents of avian viruses transferred to humans in the distant past. The influenza viruses are certainly more closely related to one another than to the two other genera in the Orthomyxoviridae; the tick-borne Thogoto-like viruses [55] and Isavirus, the infectious salmon anemia virus [67].

Two physical characters of the influenza genome allow for incredible evolutionary flexibility. First, the influenza virus has a segmented genome, bearing one or two genes per segment. This allows reassortment, or the mixing of segments, when two viruses infect a single host cell. Second, because influenza is an RNA virus, there is no proofreading mechanism to correct errors of replication. Reassortment and frequent mutation alone do not guarantee rapid evolution, but they do allow for rapid response to selection. I come back to the response to selection after first describing the influenza genome and the distribution of genetic variation among these viruses in nature.

13.2.1 Influenza Genome

The influenza genome, about 14kb in size, is composed of 10 genes carried on eight single-stranded negative-sense RNA segments (seven segments in influenza C). The influenza A genome encodes three polymerase proteins (PB1, PB2, and PA); two major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA); three structural proteins (NP, M1, and M2); and two nonstructural proteins involved in nuclear export (NS1 and NS2) [69]. The matrix genes differ somewhat between types A, B, and C. An eleventh open reading frame within the PB1 gene of some influenza A viruses codes for a protein apparently involved in host cell apoptosis [17,46]. The hemagglurinin esterase (HE) in influenza C assumes the functions of both the HA and NA. A number of good general reviews of influenza genetics and biology are available [25,70,155].

13.2.2 The Diversity of Influenza A Subtypes

A large amount of diversity exists among avian influenza A viruses [145]. This variation is categorized based on antibody recognition of the two surface glycoproteins. HA is involved in binding to host cell surface receptors. NA is an enzyme necessary for release of daughter virions from host cells. These proteins protrude from the viral envelope and are exposed to host immune defenses. Although HA is the primary target for neutralizing antibodies, antibodies against NA also may reduce occurrence and severity of illness, and possibly prevent infection if present at high titer. These rapidly evolving glycoproteins, particularly the HA, have been the object of most evolutionary studies of influenza.

To date, influenza viruses bearing 16 HA alleles and nine NA alleles have been isolated from birds (see references in [37]). Viral "subtypes" are classified according to the particular HA and NA alleles they carry, for example, subtype H3N2. A very limited number of HA and NA alleles circulate in humans, swine, and horses, as discussed below.

13.3 ANTIGENIC SHIFT AND ANTIGENIC DRIFT

On occasion, influenza A viruses carrying either intact avian influenza genomes, or genomes produced by genetic reassortment with avian viruses, infect humans. These viruses can cause severe and widespread global outbreaks of disease called pandemics if they contain a HA allele to which humans have little or no immunity, and if the viruses are easily transmitted from person to person. The most famous pandemic, the 1918 "Spanish flu," was responsible for approximately 40 million human deaths [133]. Two less dramatic pandemics resulting from the introduction of viruses carrying avian influenza A virus genes into humans occurred in 1957 and 1968, as described in more detail below. An evolutionary event involving the introduction of a completely novel antigen into a new host is referred to as an "antigenic shift." The limited host range of influenza B and C viruses apparently prevents their involvement in antigenic shift because they normally infect only humans, and thus limited opportunity exists for cross-species transfer.

A slower, more continuous evolutionary process called "antigenic drift" occurs in influenza lineages once they become established in a new host. The RNA polymerase complex makes errors during replication of viral genes; one estimate is $10^{-5.5}$ mutations/site/replication [147]. Because RNA viruses lack a proofreading mechanism, mutations accumulate [97]. Over time, the accumulated replacements alter the shape and the electric charge of viral surface antigens, particularly on the exposed distal surface and around the receptor binding site of the HA [24]. These cumulative changes, many of which occur in known antibody binding sites [152], eventually allow escape from antibodies raised by prior infection or vaccination [7,89,151].

Reassortment among viruses within subtypes may also contribute to antigenic drift by producing viruses whose fitness varies for reasons other than antibody recognition of surface proteins. Reassortment has been observed within and between influenza A subtypes, and within influenza types B and C [3,4,75,102,112,156]. There is no evidence of intra-typic reassortment or recombination between types A, B, and C.

Recombination has not been thought to play a major role in influenza evolution. However, co-circulating influenza viruses are typically very closely related to one another, and thus it might be difficult to recognize recombination unless it resulted in fitness effects with epidemiological consequences. Recombination was recently implicated as a factor in the emergence of several highly pathogenic H7N3 avian strains in Chile and Canada [98,128]. It has been suggested that the HA gene of 1918 pandemic strain originated through recombination [47], but this was more likely a reflection of error in phylogenetic reconstruction [154].

13.4 HOST SPECIFICITY

Farmers in Southeast Asia have long been reported to carry antibodies to a number of avian influenza subtypes not known to circulate in humans, including the H5 allele currently

involved in outbreaks of human illness in Southeast Asia [115]. However, prior to the deaths of six humans in Hong Kong in 1997 from avian influenza, many had thought that the direct infection of humans by avian viruses was highly restricted. This belief was based on laboratory experiments which suggested that avian viruses show preferential binding to sialic acid molecules linked to galactose via α2,3-glycosidic bonds. These molecules are found on cells in the avian intestine. Human tracheal epithelia have α2,3 linked galactosidases [61,118].

Swine lung cells contain both the cell receptors preferred by avian and human influenza viruses, and as a consequence swine have been proposed as necessary intermediate hosts or "mixing vessels" for the transmission of avian viruses to humans [111]. This hypothesis is consistent with the observation that many influenza epidemics and pandemics appear to originate in Southeast Asia, where agricultural practices put ducks, swine, and humans in close contact [30].

However, there is no evidence that any known human outbreaks have been caused by swine-adapted influenza. Even the so-called "swine flu" epidemic in 1976 among military personnel at Fort Dix, which prompted a mass vaccination program in the United States, was ultimately a short-lived outbreak [31]. Reanalysis of the Scholtissek data [111] has also failed to convincingly support the hypothesis that pig served as intermediate hosts in the emergence of the 1918 pandemic strain [10].

Evidence for direct infection of humans by avian viruses does not prove that swine have never been involved in the transmission of avian influenza to humans. It suggests, however, the existence of additional barriers to establishment of avian viruses in mammals above those imposed by receptor preference. Dehydration during aerosol transmission among humans, for example, is a challenge not experienced during spread in feces and in the aquatic environments of waterfowl. We know almost nothing about the biology or genetics of influenza transmission.

In early 2003, a highly pathogenic strain of avian influenza A subtype H7N7 caused an outbreak in chickens in the Netherlands [38]. This virus was subsequently detected in 86 people. Their most common symptom was conjunctivitis. As several ocular human pathogens bind α2,3-galactosidase, this observation led to speculation that the human eye might serve as a site in which avian viruses could begin the process of adaptation to the α2,6 receptors in the human lung [94]. Although the eye could potentially serve as an evolutionary midpoint between a bird gut and the human lung, it does not seem to be a necessary one, as conjunctivitis is not a common symptom in current infections of humans by H5N1 avian viruses.

There are many types of sialic acids. We know very little about how the subtle differences between these variants affect viral binding. We also know little about the distribution of potential receptor molecules among the organs and tissues of different animals in nature. These are important areas for future research (for reviews see [83,130,161]) as is the biology of post-adhesion events such as host cell entry and membrane fusion [20,117].

13.5 AVIAN INFLUENZA

The natural hosts of influenza A infections are waterfowl, gulls, and shorebirds. Infections in these birds are typically intestinal and asymptomatic. Transmission of avian influenza into domestic poultry, such as chickens and turkeys, may result when infected waterfowl visit open-air poultry farms or are exposed to poultry in the market [148]. A variety of subtypes and evidence of reassortment among influenza A strains have been found in fowl sold in the live animal markets in Southeast Asia [19,78,91]. Most of our knowledge of genetics and population biology of avian influenza comes from studies of domestic poultry [60,146].

Symptoms of avian infection in domestic poultry vary by viral subtype. Most infections, caused by so-called "low-pathogenicity" strains, cause symptoms similar to infections in natural hosts. That is, these viruses are carried asymptomatically and replicate in the cells lining the intestinal tract [62]. A few influenza A subtypes (particularly H5 and H7) may cause systemic disease with central nervous system involvement and rapid progression to death [155]. These are called "high-pathogenicity" or "fowl plague" strains.

A key difference between low- and high-pathogenicity avian influenza viruses is the presence of multiple basic amino acids at the HA cleavage site [139]. Viral infectivity depends critically on cleavage of the HA precursor protein (HA0) into two subunits, the HA1 and HA2 domains. Cleavage of the HA0 by host proteases is necessary for fusion of the viral envelope with host endosomal membranes [150]. The presence of additional basic residues allows cleavage by ubiquitous proteases such as furans, which are widely distributed throughout the bodies of animals. The presence of these basic residues in the HA cleavage site is correlated with increased virulence and viral replication in organs and tissues other than those typically infected in birds. Highly pathogenic avian influenza strains can evolve from low-pathogenicity strains by a few point mutations in the HA gene; the accumulation of such residues in an evolutionary lineage over time is thus a cause for concern (for reviews see [2,58,124]).

Despite the importance of avian influenza to public health, the incidence and frequency of disease caused by the different A subtypes have not been extensively documented in wild birds. Stallknecht [122] found influenza A in 90 species representing 12 orders of birds, primarily shorebirds and waterfowl [123]. Studies of Canadian ducks [56] and of ducks and shorebirds in North America [66] show pronounced geographical and temporal variation in subtype frequency and overall carriage. Avian infections were typically asymptomatic, with higher incidence in juveniles [66].

Phylogenetic analysis suggests that both geography and host taxonomy play key roles in generating the observed patterns of genetic variation in avian strains [145]. Phylogenetic trees depicting the evolution of avian influenza differ somewhat depending on the gene employed. For example, using PB1 sequences it appears that avian viruses

isolated from North American waterfowl are most closely related to viruses from gulls. In contrast, trees constructed using the NP, M, and PA genes show a closer relationship between viruses from North American waterfowl and Old World waterfowl [49]. More recent studies also show some evidence of large-scale (continental) geographic structure but with evidence of reassortment between regions as well [140,149]. The differences between gene trees may be attributable to genetic reassortment, which occurs frequently between avian strains, and also from the small number of sequences used in these analyses.

13.6 SWINE AND EQUINE INFLUENZA

Introductions of both avian and human influenza strains into swine occur fairly frequently. Influenza viruses cause acute respiratory disease in swine and are endemic in swine populations worldwide. The first documented swine influenza epidemic occurred concurrently with the 1918 H1N1 pandemic in humans. The descendants of that outbreak, referred to as "classical" swine H1N1 influenza viruses, are a major source of disease in swine in North America to this day. In addition to the classical H1N1 viruses, currently circulating swine strains include more recently introduced avian H1N1 strains, human H1N1 and H3N2 strains, and reassortants among these strains [9,80,142].

Viruses belonging to influenza A subtypes H3N8 and H7N7 have caused both limited and decades-long outbreaks of acute respiratory disease in horses [81,86]. Only H3N8 circulates today. Although an economic problem, particularly within the racing industry, equine influenza has not been of concern with respect to human health.

13.7 HUMAN INFLUENZA

I first discuss the evolution of the human-adapted influenza strains responsible for the familiar winter epidemics of the north and south temperate zones. I then turn to pandemic influenza, a rare and much more serious event.

13.7.1 Epidemic Influenza

Influenza epidemics are widespread outbreaks of highly contagious respiratory disease that appear suddenly, persist for a few weeks, and then just as suddenly disappear. Symptoms of infection appear abruptly and can persist for 1–2 weeks. In adults, viral replication typically peaks at about 48h post infection, declines slowly thereafter, with little viral shedding after 6–8 days. Attack rates during influenza epidemics are generally 10–20%, and are often highest in young children. Influenza infections range in severity from asymptomatic to lethal. Death is most common in the elderly or those with compromised cardiovascular or immune systems, and is typically associated with a secondary bacterial pneumonia or exacerbation of underlying health conditions [26].

Influenza is said to be epidemic when the incidence of influenza-related illness rises above a seasonal baseline. This is illustrated using data on influenza and pneumonia-related (P&I) deaths, as in Figure 13.1. Winter outbreaks occur yearly in both the north and south temperate zones, but influenza can be isolated from humans somewhere in the world year round [87]. There is substantial temporal and geographic heterogeneity in influenza incidence. Thus, Figure 13.1, which depicts P&I mortality data for the United States, may not accurately portray influenza deaths worldwide.

It has long been known from immunological studies that more than one type or subtype of human influenza can

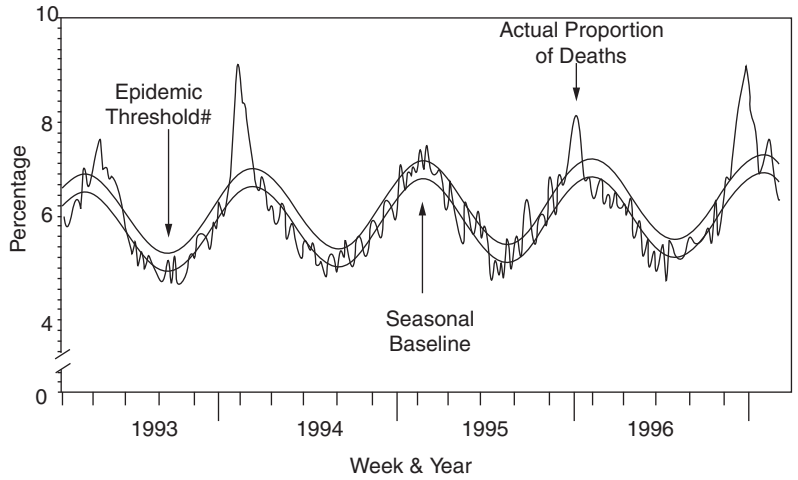

Fig. 13.1. *Influenza mortality.* Weekly pneumonia and influenza (P&I) mortality as a percentage of all deaths in 122 cities – United States, January 3, 1993–April 5, 1977. The epidemic threshold is 1.645 standard deviations above the seasonal baseline. The expected seasonal baseline is projected using a regression procedure in which a periodic regression model is applied to observed percentages of death from P&I since 1983. From *Morbidity and Mortality Weekly Reports* **46**(5):327.

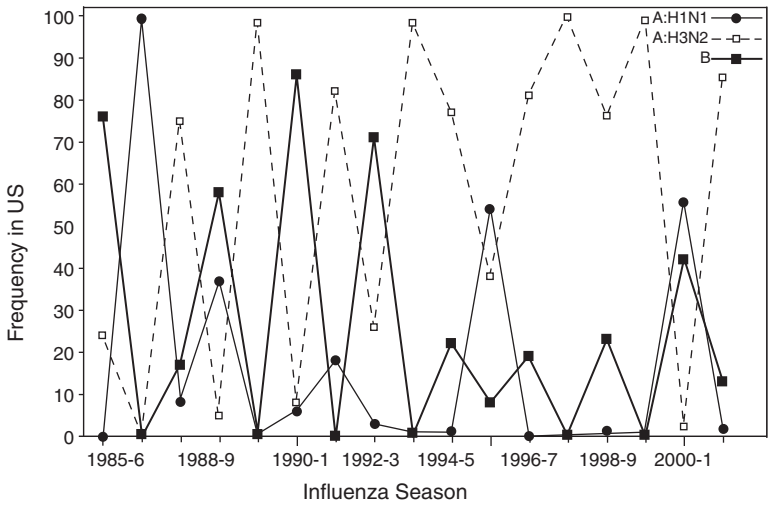

Fig. 13.2. *Frequencies of influenza strains.* The relative frequencies of influenza B and influenza A subtypes H3N2 and H1N1 in the United States.

circulate during an influenza season. Figure 13.2 shows the relative frequency of the three influenza strains currently circulating in humans (H1N1, H3N2, and B) in the United States between 1985 and 2001. In many years, one strain is predominant, but in the 2001–2002 influenza season (October, 2001 to September 30, 2002), for example, influenza A subtype H1N1 and influenza B viruses were reported at similar frequencies.

Over short intervals of time, there may appear to be a regular periodicity in the order in which the types or subtypes predominate, such as during the first 10 years shown in Figure 13.2. However, on examination of longer periods of time (looking across Fig. 13.2), these patterns disappear. These data are again only for the United States; substantial geographic variation occurs. For example, in the 1985–1986 season, influenza B was the predominant influenza virus reported in the United States; elsewhere in the world, influenza A H3N2 viruses were most commonly reported.

Comparison of Figures 13.1 and 13.2 shows that although there is no exact correlation between disease severity (Fig. 13.1) and the type or subtype of influenza in circulation (Fig. 13.2), there is a correlation between the predominance of influenza A subtype H3N2 viruses and the severity of the season. For example, seasons with the highest peaks of excess P&I deaths (1993–1994 and 1996–1997) were those in which H3N2 viruses predominated. These figures also show, however, that the number of P&I deaths cannot be predicted solely on the incidence of a particular subtype. For instance, the incidence of influenza-related disease in 1994–1995 barely exceeded the seasonal baseline, but was at epidemic levels for over 2 months in 1996–1997 (Fig. 13.1). Yet the relative frequencies of the three strains that circulated in the 1994–1995 and 1996–1997 influenza seasons were almost identical (Fig. 13.2).

The above example provides a nice illustration of why both the accumulation of host immunity to different circulating strains and the ongoing evolution of those strains are important in determining the course of antigenic drift. Influenza A viruses antigenically similar to the H3N2 reference strain A/Johannesburg/33/94 circulated in both the 1994–1995 and 1995–1996 seasons. This presumably resulted in widespread immunity in the human population. The epidemic of 1996–1997 was caused by an H3N2 virus that had evolved from an ancestor of the A/Johannesburg/33/94-like strains. These newly evolved viruses, related to the A/Wuhan/359/95 reference strain, were not effectively neutralized by antibodies against A/Johannesburg/33/94, leading to increased incidence of H3N2 in the in the 1996–1997 season.

Antigenic drift occurs in direct response to the changing antibody profile of a continually reinfected human population. Studies of influenza evolution typically focus on genetic change in the virus, essentially ignoring one half of this coevolutionary system. Unfortunately, there have been few studies of changing immunity in human populations in response to antigenic drift (see review in [119]). Thus, we have little option but to use viral data as our primary source of information on influenza evolution.

13.7.1.1 Phylogenetics Phylogenetic trees constructed using influenza sequence data are a natural way to monitor the genetic changes associated with antigenic drift. The World Health Organization (WHO) Global Influenza Surveillance Network has conducted influenza surveillance worldwide since 1952. Antigenic characterization initially formed the basis of surveillance; gene sequencing was added as a surveillance tool in the mid-1980s. These efforts have produced a wealth of data for evolutionary analysis.

Figure 13.3 shows phylogenies constructed using the HA1 domain of the HA gene of human influenza A subtypes H3N2 and H1N1 and of influenza B [12]. The slender "trunk" of the H3 tree in an especially characteristic reflection of the serial replacement of strains that results from antigenic

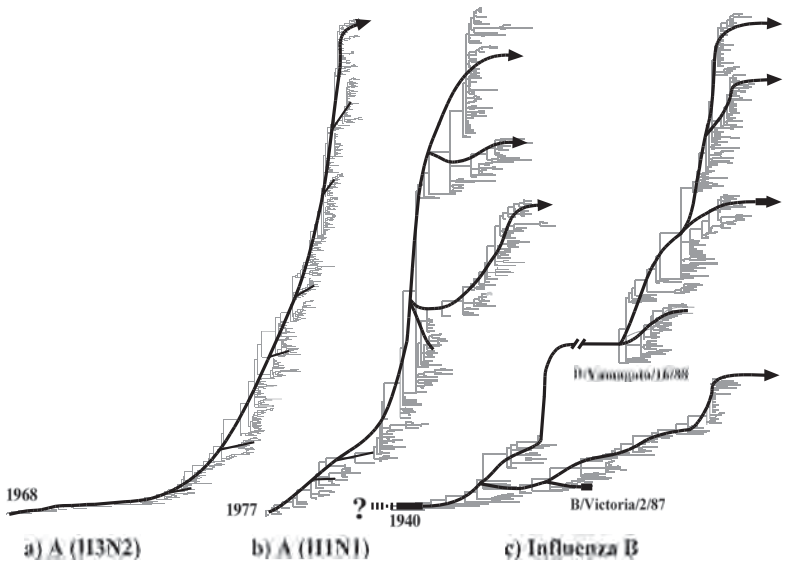

Fig. 13.3. *Influenza phylogenies*. Phylogenetic trees showing the evolution of the HA1 of influenza A subtypes H3N2 (a) and H1N1 (b) and influenza B. Dark lines are superimposed to indicate some of the major genetic lineages. As explained in the text, because of non-random sampling the relative size of various branches is not an accurate reflection of the frequency of those genetic lineages in nature. Lines ending in arrows indicate extant lineages.

drift. Although brief co-circulation of closely related H3 lineages is not uncommon, the H3 tree appears linear because the average survival time of side lineages is only 1.6 years [36]. The H1 tree is similar to that of H3, although recent bifurcation of the main trunk has resulted in several extant lineages. It remains to be seen whether more than one will persist.

The influenza B virus was first isolated from humans in 1940; its evolutionary history prior to that point in time is unknown. Although limited sequence data exist prior to the mid-1980s, phylogenetic analysis show a split into two distinct B lineages sometime in the early 1970s. One lineage circulated globally throughout the 1990s; the other lineage was restricted to Asia. In the last few years, the restricted lineage has reappeared. Reemergence may have been due to the accumulation of a large cohort of immunologically naive children. However, genetic change, including between-lineage reassortment, occurred during this time as well [157]. If both influenza B lineages persist, it may be necessary to add an additional influenza B virus to the current vaccine formulation.

The processes responsible for these phylogenetic patterns are not well understood. Ferguson et al. [35] studied the interaction between ecological and immunological factors affecting drift using a mathematical model that incorporated both realistic epidemiological dynamics and viral evolution at the sequence level. Matching of model output to phylogenetic patterns suggested that short-lived strain-transcending immunity, such that infection by one strain of influenza prevented reinfection by any strain during the next month or so, was essential to restrict viral diversity and linearize the tree. This result is intriguing, but the precise immunological factors responsible remain to be identified.

It is easy to draw intuitive but incorrect inferences from quick visual inspection of an influenza tree. For instance, one might think, given the linear phylogeny of the H3N2 tree, that each subsequent influenza epidemic evolves from the previous epidemic strain. This is not always the case. For example, the A/Singapore/1/86-like H1N1 viruses that caused the 1986–1987 epidemic did not evolve from the previous epidemic strain, the A/Chile/1/83-like viruses. They belonged to a separate lineage; the ancestor of both strains was a virus that had circulated much earlier in China.

It is also a mistake to assume that the sizes of the clades (genetic clusters) on the trees in Figure 13.3 reflect the frequencies of these genotypes in nature, or the severity of the disease that they caused. Surveillance by the WHO, currently the main source of influenza sequence data, is purposefully biased toward sequencing viruses that differ antigenically from the current epidemic strain on the basis of the hemagglutination inhibition test. As a result of this intentional sampling bias, HA genotypes at high frequencies in nature may in fact not be most frequent in GenBank, particularly in years with few samples. Assuming otherwise [103] will inevitably lead to erroneous results under current WHO sampling protocols.

13.7.1.2 Rates of evolution Most studies suggest that influenza A subtype H3N2 evolves more rapidly than H1N1, whereas influenza B evolves more slowly than either A subtype [23]. Fixation rates calculated recently by [35] for H3N2, H1N1, and B were consistent with this at 0.0037, 0.0018, and 0.0013 nucleotide substitutions per site per year, respectively. However, rate estimates vary considerably among studies.

Some of this variation may reflect the short time periods under study [23,27]. This has been illustrated in some detail for the H3N2 subtype of influenza A [10]. The fixation rate of influenza A in swine, which like humans are a novel host for influenza A, is similar to the rates seen in humans [113].

The fixation rate of influenza A in its natural avian host is much slower than in humans or swine [145]. This may be due to the shorter life span of birds and the relative lack of immune selection during viral replication in birds. However, avian influenza viruses introduced into domestic poultry, which are not natural hosts of influenza, show fixation rates similar to those seen among human influenza viruses [127].

Phrases such as "evolutionary stasis" and "frozen evolution" are sometimes used to describe the relatively low rates of influenza evolution seen in birds and horses [6,144]. Such terms have no precise meaning and unfortunately give the impression that evolution does not occur at all in these lineages.

13.7.1.3 Genetic basis of antigenic change Influenza surveillance based on HA1 nucleotide sequences provides more detail on evolutionary change in circulating strains than does traditional antigenic analysis. However, the usefulness of sequence data for epidemic prediction is limited because the relationship between genetic and antigenic variation is often unclear. The evolution of a new "drift variant" in humans appears to require at least four amino acid replacements occurring in at least two of the five antigenic sites in the HA1 of H3N2 strain [24]. However, there is a great deal of variation in this pattern from year to year. One way to more directly address the question of how genetic change relates to antigenic change is to use evolutionary hypotheses to try to anticipate the fitness of particular types of new mutant strains.

It has been shown that a small number of codons in the HA1 of influenza A subtype H3N2 have been under positive selection in the past to repeatedly change the amino acid they encode. These codons show an excess of non-synonymous (amino-acid changing) versus synonymous (silent) nucleotide substitutions, a pattern consistent with the assumption that host antibody pressure drives change in HA conformation. Analysis of phylogenetic trees representing the evolution of the HA1 of human H3N2 viruses through a short period of time (1987–1997) showed that strains with a greater number of new mutations in a set of 18 codons with a history of excess non-synonymous substitutions were the progenitors of successful new lineages in nine of 11 recent influenza seasons [11,13]. A causal explanation for these results was suggested by the physical location of these codons in the HA1. Most occur in or near two important antibody binding sites, and some surround the receptor-binding pocket as well.

Future progress in this area will most likely come from the use of plasmid-based reverse genetics. This is a methodology that allows the generation of influenza viruses entirely from cloned cDNA [44,90]. Briefly, human embryonic kidney cells are transfected with eight plasmids, each encoding the viral RNA of an influenza virus. Transcription of viral genes by cellular RNA polymerase I produces all eight influenza viral RNAs. This process requires a functional ribonucleoprotein complex because neither genomic viral RNA nor complementary RNA of negative-sense RNA viruses can serve as a direct template for protein synthesis. Cotransfection with protein expression plasmids for the nucleoprotein and polymerase proteins yields infectious virus.

Smith et al. [120] recently presented an application of this technology to testing evolutionary hypotheses about influenza evolution. They first developed a method for constructing two-dimensional maps based on antigenic distances between viruses, as measured by the hemagglutination inhibition assay. Comparison of antigenic and genetic maps identified a case in which a single amino acid replacement in the HA separated two antigenic clusters. Site-directed mutagenesis confirmed the effect of this replacement on antigenicity. Although technically still challenging, reverse genetics will be increasingly used to directly investigate the effects of genetic change on viral fitness [108].

13.7.1.4 Challenges in studying drift The existing influenza sequence data are among the best available for studying the evolution of an infectious disease. However, there are problems with using these data to study influenza evolution and population biology. First, the bias inherent in the influenza surveillance system toward purposefully sequencing viruses that differ antigenically from commonly sampled strains on the basis of the hemagglutination inhibition test causes an overestimation of positive selection on the HA gene because only non-synonymous mutations produce antigenic change [15].

Another problem is the presence of laboratory artifacts in the sequence data. Although cell culture is increasingly used, amplification of the virus by passage in embryonated hens' eggs has been standard laboratory practice for the culture of influenza viruses for many years. Unfortunately, the HA1 of human influenza viruses evolves rapidly to adapt to replication in eggs [110]. The resulting sequences may thus contain replacements that were either not present or were at low frequency in the original viral sample. These laboratory artifacts often occur at sites involved in adaptation to humans as well as to eggs [24].

It is possible to estimate the proportion of amino acid replacements resulting from egg passage by contrasting the number of replacements found in sequences in cell-passaged and egg-passaged isolates [14,15]. In the HA1 domain of influenza A subtype H3N2, egg passage of human strains was associated with about 8% of amino acid replacements [13]. Unfortunately, in the absence of controls – viruses that have never been passaged – it is impossible to determine which replacements in a data set are artifacts.

Non-synonymous substitutions due to egg passage should be eliminated from analyses seeking evidence of selection by the human immune system because these changes do not result from coadaptation with the human host. One way to minimize error due to these artifacts when doing evolutionary studies is to discard changes assigned to the terminal branches of the trees [13–15]. Studies that have failed to

exclude egg-passaged replacements routinely find evidence for selection on codons for which there is no evidence of a selective advantage in humans [59,92,159, 160].

Much research on the immunological response to influenza infection, particularly with respect to vaccine and drug development, has been done in mice. Mice are not natural hosts of influenza; viral strains must be serially passaged to adapt for easy infectivity in mice [116]. Because influenza viruses are typically identified by their original isolate name no matter how they were later handled, care must be taken to differentiate passaged and original isolates. Human influenza strains are named by type/location/collection number/year. Thus, A/Chile/1/83 was the first influenza type A virus isolated in Chile in 1983. Although nomenclature can be irregular, swine viruses typically begin with A/sw/, equine viruses with A/eq/, and avian viruses with either A/avian or the common name of the host bird, that is, A/chicken/.

13.7.2 Pandemic Influenza

Influenza pandemics are characterized by the rapid worldwide spread of a virus containing an HA and sometimes a NA to which humans have had no previous exposure. Pandemics result in high rates of morbidity and mortality, social disruption, and economic loss, although the cumulative morbidity and mortality during inter-pandemic years due to epidemic influenza actually exceeds that of pandemics [25].

Historical records describing influenza pandemics go back hundreds of years, but we have documentation for only a few pandemic events [104]. Pandemics seem to occur as several waves of severe disease. The causal viruses then apparently evolve to a lower level of virulence, and subsequently reappear annually as typical winter epidemic strains. There have been only two opportunities (1957 and 1968) to observe pandemics since the human influenza A virus was first isolated in 1933, both occurred prior to the advent of molecular biology [121]. However, sequencing of archived viruses has allowed us to partially reconstruct the genetic changes associated with the three most recent pandemics.

13.7.2.1 1918 H1N1 "Spanish flu" The 1918 influenza A subtype H1N1 pandemic occurred in waves of increasingly virulent disease starting in the spring of 1918 and continuing through the subsequent winter. This pandemic resulted in the deaths of approximately 40 million people worldwide. Although called "Spanish flu," this virus' geographic origin is controversial [96]. The transmissibility of the 1918 pandemic strain may not have been unusually high [84], however, it had a high per-case mortality rate, and an unusually high relative rate of death in young adults. The manner in which many deaths occurred was also unusual. Influenza-related mortality typically occurs a week or two post infection, and is associated with a secondary bacterial pneumonia or other complications. In 1918, many people died within just a few days from virally induced hemorrhagic pulmonary edema [132].

Recent investigations of the 1918 pandemic strain have yet to reveal its origin, or why it was so deadly [107]. Taubenberger and colleagues [105,134] have studied the 1918 pandemic strain by sequencing viral genes recovered from the archived lung tissue of two army soldiers and from an Alaskan Inuit woman frozen in permafrost, all victims of the 1918 pandemic. X-ray crystallographic studies of HA proteins reconstructed from 1918 sequence data suggest that the binding site of the 1918 human virus is more avian-like than that of recent human H1N1 viruses [42,125].

However, phylogenetic analysis suggests that if the 1918 human pandemic strain was avian derived, it must have evolved undetected in a non-avian host for some time prior to the 1918 human pandemic [106]. Whether a pig was involved is unknown. The HA and NA resemble the oldest available classical H1N1 swine influenza strains (from 1930), but share characteristics with modern avian H1N1 viruses as well. Sequences from viruses isolated from waterfowl collected in 1917 and preserved in alcohol in the American Museum of Natural History have done little to resolve this mystery [34,106]. The paucity of archived viral isolates from human, swine, and avian hosts may prevent us from ever determining the sequence of events that resulted in the 1918 pandemic.

Reverse genetics has recently been used to investigate the cause of increased virulence in the 1918 H1N1 pandemic strain [65]. Human viruses that were otherwise nonpathogenic in mice showed enhanced virulence when carrying the 1918 HA. High chemokine and cytokine levels resulted in infiltration of inflammatory cells and severe hemorrhage throughout the mouse lung, symptoms similar to those seen in humans during the 1918 pandemic.

Several studies suggest that the NS1 protein affects influenza virulence by blocking the interferon response, and that this effect may have affected virulence of the 1918 H1N1 pandemic strain and recent H5N1 viruses as well [5,43,45,76,114]. However, other experiments found recombinant viruses containing both 1918 HA and NA genes to be highly lethal in mice, whether or not an NS gene was included [136]. And in earlier work, the addition of a 1918 NS gene resulted in the attenuation of a human strain that was otherwise highly virulent in mice [135].

The lack of consensus among these recent studies of the effects of NS1 on pathogenicity may only reflect differences in the animal host and cell types used [76]. This is an exciting area of research, still in its infancy, that should in coming years reveal much about how the influenza virus functions and evolves.

13.7.2.2 1957 H2N2 "Asian flu" and 1968 H3N2 "Hong Kong flu" In 1957, an H1N1 strain circulating in humans – a descendent of 1918 pandemic strain – obtained new H2, N2, and PB1 genes through reassortment with an avian influenza A virus [63]. The resulting H2N2 strains caused a pandemic that began in February of 1957 in China and by summer had spread worldwide. A second wave of disease occurred in the winter of 1958. Total influenza-related

excess mortality in the United States was estimated at 70,000 [93]. With the spread of the H2N2 strain, the parental H1N1 lineage disappeared from circulation in humans. After the initial waves of pandemic disease, H2N2 continued to circulate as a typical winter epidemic strain until being itself displaced by the next pandemic.

The 1968 H3N2 "Hong Kong flu" pandemic was caused by a human-adapted H2N2 virus that obtained avian H3 and PB1 genes through reassortment [63]. Phylogenetic analysis of H2N2 viruses isolated between 1957 and 1968 and of H3N2 viruses from 1968 through 1972 showed that the establishment of H3N2 viruses in humans resulted after multiple reassortment events between circulating H2N2 strains and reassortant viruses carrying the new avian H3 and PB1 alleles [75].

The origin of the 1957 H2N2 pandemic strain may also have involved multiple reassortment events. Unfortunately, we have very few humans samples, and virtually no avian and swine viruses, from that time period. This speaks strongly for the preservation of existing museum collections from which viral RNA can be extracted, and for expanded surveillance and archival of extant avian and swine strains.

The H3N2 pandemic resulted in only about half as much mortality in the United States as the H2N2 pandemic, perhaps because the human population already had antibodies to the N2 allele [93]. The fact that the HA and PB1 genes reassorted together in both 1957 and 1968 suggests some type of constraint or dependency that we do not understand. Or this may only have been a coincidence.

13.7.2.3 1977: the reappearance of H1N1 In 1977, human-adapted H1N1 viruses mysteriously reappeared in northern China 20 years after they last circulated in humans. These viruses, virtually identical to H1N1 human viruses from 1950, were most likely preserved during the intervening decades in a laboratory freezer [88]. The reemerged H1N1 strain spread rapidly but caused relatively mild disease [22]. Illness occurred almost exclusively among persons under 20 years of age, illustrating that some level of immunity against the H1N1 strain had persisted for 20 years.

This incident inadvertently shed some light on the question of whether pandemic viruses really do evolve to lower levels of virulence. All documented pandemic strains produced decreased severity of infection after the first few waves of disease. This pattern might reflect the accumulation of antibodies in the host population that are broadly protective against each new subtype. However, symptoms in young adults infected by the H1N1 virus in 1977 were very mild, whereas its ancestor, the 1918 H1N1 pandemic strain, had been deadly. Thus, it may be safe to assume, in at least this case, that evolution to a lower level of virulence had occurred.

Another interesting aspect of the 1977 H1N1 reemergence was that the influenza A subtype then in circulation, H3N2, did not subsequently disappear. With the 1918, 1957, and 1968 pandemics, the introduction of a new influenza A subtype into humans was in each case quickly followed by the extinction of the previously circulating strain. It had thus been suspected that, for whatever reason, only one subtype of influenza A could circulate in the human population at any point in time. Influenza A subtypes H1N1 and H3N2 have co-circulated in humans for the last 28 years.

Modeling has shown that short-lived nonspecific immunity might be responsible for limiting the within-subtype diversity of influenza A strains [35]. Such immunity could also be involved in subtype replacement. If spread of new pandemic strain into an immunologically naive population inhibits immediate reinfection by the previously circulating subtype on a global basis, the previous subtype may lack sufficient susceptible hosts and become extinct. However, where subtype introduction causes a smaller epidemic, as in the reemergence of H1N1 in 1997, coexistence could be possible if the duration (or effectiveness) of nonspecific immunity is greater within subtypes than between subtypes, a conclusion for which there is abundant empirical evidence [48].

Reassortment between the H1N1 and H3N2 influenza viruses circulating in humans (and swine) has occurred repeatedly. Most human H1N2 reassortants have not persisted for very long, and resulting disease was not particularly severe [24,54]. In the 2001–2002 influenza season, a new H1N2 reassortant spread across a wide geographical area [51] and remains in circulation today. The current vaccine contains both the H1 and N2 alleles and thus should provide adequate protection as long as the H1N2 reassortant does not drift too far from the H3N2 and H1N1 strains in the vaccine [157].

13.7.2.4 Seroarchaeological evidence of historic pandemics The nature and duration of the human immune response to influenza is only partially understood. A variety of immune mechanisms are likely to be involved. However, immunity appears to be subtype and strain specific, and primarily mediated by antibodies against the HA and NA glycoproteins. For unknown reasons, the first variant of a subtype encountered by an individual typically causes the strongest antibody response. Subsequent infections with related variants tend to reinforce the response to the first variant. Thus, the highest antibody titers in an age group tend to reflect the dominant antigens of the virus responsible for the childhood infections of the group. This phenomenon is called "original antigenic sin," [40].

In the mid-1900s, older people were screened for antibodies to a number of HA antigens. The results suggest that what was thought of as an unusually high rate of mortality in young adults in the 1918 pandemic might be better described as an unusually low mortality rate among the middle aged and elderly. Older people in 1918 appeared to be at least partially protected by antibodies, presumably produced in response to exposure to an H1 allele in the late 1800s.

Serum collected before the H2N2 or H3N2 subtypes entered the human population in 1957 and 1968, respectively, suggest that older human cohorts had also previously been exposed to H2 and H3 alleles. Thus, we have evidence that

the H1, H2, and H3 have all circulated in humans at least twice in recent history. This does not guarantee, however, that viruses carrying other HA alleles cannot become established in humans. For a review of seroarchaeology see [32].

13.8 THE CURRENT AVIAN H5N1 OUTBREAK

Outbreaks of disease caused by avian H5N1 viruses erupted in populations of both poultry and humans in Hong Kong in 1997. Six of the 18 known humans cases resulted in death [21,29,129]. These H5N1 viruses appeared to be reassortants containing a mix of genes from viruses found in goose, teal, and quail, all birds commonly housed in close proximity in the live animal markets of Hong Kong [53,57,158].

In response to this outbreak, all domestic poultry in Hong Kong were killed. The initial optimism over the apparent success of this intervention was short lived. Viruses with the exact genome of the 1997 H5N1 strain have not reappeared, however, individual genes from this strain have continued to circulate and reassort in domestic poultry across Southeast Asia [52].

In early 2003, outbreaks of a new strain of H5N1 occurred in poultry. The HA and NA genes of these viruses, referred to as the "Z" strain, are descendants of the 1997 H5N1 viruses, but the internal genes are not [74,91,141]. This highly pathogenic strain continues to cause disease in poultry throughout Southeast Asia. Known human deaths resulting from contact with H5N1-infected birds currently stands at 54 [100].

Avian H5N1 strains are being monitored for additional evidence of reassortment. Also under surveillance are H9N2 avian viruses, which contributed some of the RNA segments to the 1997 H5N1 strain [53]. H9N2 influenza viruses circulate widely in domestic poultry in Asia, and have caused transient infections in both humans and pigs [18,101].

An ominous sign in any avian influenza virus is change in the shape of its receptor-binding pocket that suggests adaptation to humans. Although current H5N1 viruses possess avian-like binding pockets, their affinity for sialic acids linked via the α2,6 bonds typical of human cell receptors can be increased experimentally through a very limited number of mutations [82].

Another fear is that the H5N1 strains will evolve expanded tissue tropism. There has been little to suggest that H5N1 viral replication occurs outside of the respiratory tract in humans despite the presence the avian-like string of basic residues in the HA cleavage site. However, one recent study found evidence of H5N1 replication in the intestine of a child on autopsy [137]. The significance of this single finding is not clear – surprisingly little information is available on the pathology of avian influenza viruses in humans. Autopsy is not traditionally practiced in most countries affected by avian influenza. In addition, diagnosis of avian influenza is often not made until after death or heavy treatment with antiviral drugs, both of which make pathological examination of viral material problematic.

Thus far, there has been very limited evidence suggesting human-to-human transmission of H5N1 strains [8], although some think that this may be changing [138]. We desperately need to learn more about the biology and mechanics of influenza transmission, but it impossible to study such a deadly virus in humans. Nonhuman primates are a possible alternative; macaques infected with avian H5N1 influenza A develop a necrotizing pneumonia similar to that seen in humans [109]. However, the expense of studying transmission with these animals is formidable, and in some eyes, unethical.

The most common animal models for the study of influenza are mice and ferrets. Transmission studies will most likely proceed in ferrets, in whom symptoms of infection [131], unlike those of mice [116], are remarkably similar to those seen in humans. But no matter what the choice of animal model, there is no guarantee that the results will apply well to humans.

H5N1 viruses have been involved in several incidental or induced infections of animals not typically exposed to avian influenza in nature, including tigers, leopards [64], and domestic cats [68]. It is not clear that these observations are of biological and thus epidemiological significance. Much more worrisome is range expansion of highly pathogenic H5N1 viruses into populations of wild birds.

There have been several recent reports of H5N1 viruses infecting wild birds in Southeast Asia including flamingos, egrets, herons, and wild ducks [33,126]. Although the death of these birds is in itself a concern, more worrisome with respect to public health is the potential for migratory wild birds to transport pathogenic H5N1 viruses outside the current epidemic area. Surveillance is insufficient to determine the extent of natural spread by wild birds. In contrast, the transport and sale of infected domestic stock by humans is known to be a problem. Eradication of disease from small backyard poultry flocks in the affected area has proved impossible because rural families depend on these birds for income. The best current source of information on the sociological aspects of the avian influenza outbreak is ProMED (http://www.promedmail.org), an electronic outbreak reporting system that monitors infectious diseases globally.

13.9 EVOLUTION AND INTERVENTION

Vaccines are currently available for the prevention of human influenza and also for some subtypes of avian influenza. Two classes of antiviral drugs, although not widely used, have been approved for influenza prophylaxis and treatment. I briefly describe the impact of influenza evolution on vaccine and antiviral efficacy, and end by discussing current plans for intervention should avian H5N1 influenza viruses evolve the capability to spread among humans.

The most common intervention for human epidemic influenza is prophylactic vaccination. The human influenza vaccine, first developed in 1947, is currently a trivalent formulation containing one influenza B virus and one virus

each from influenza A subtypes H3N2 and H1N1. Antigenic drift requires that vaccine strains be updated almost yearly. Selection of viral strains for inclusion in the vaccine is based on antigenic, genetic and epidemiological data, and practical issues such as the availability of high-growth reassortants suitable for vaccine production.

It takes about 8 months to proceed from viral strain selection to vaccine delivery. The main factor limiting the speed of production is the requirement that the virus be grown in chicken eggs. Reverse genetic and cell culture techniques are now being applied to the production of an H5N1 vaccine. If successful, these methods may eventually be adopted for production of the yearly vaccine as well. This would provide greater flexibility and standardization in vaccine production, and also allow for a more rapid vaccine response to antigenic drift.

The need for frequent vaccine updates might also be reduced by the development of a vaccine eliciting protective antibodies against a more evolutionarily stable part of the virus than the HA. Constructs containing the conserved domains of the influenza A M2 matrix protein show promise in mice but have yet to be tested in humans [28,39]. Rapid evolution of M2 escape mutants has been seen in vaccinated mice [162]; however, HA and NA escape mutants also arise quickly under immune pressure in the laboratory.

Vaccination against influenza is infrequent in developing nations, and uncommon even in developed countries, where the target populations for vaccination are the elderly and infirm. In 2000, about 235 million doses of influenza vaccine were used. This is enough to cover about 4% of the global population (http://www.who.int). Although we have no direct evidence to address this issue, human vaccination at such low rates probably exerts a negligible selective force on influenza evolution.

Mass vaccination against human-adapted influenza viruses has not been recommended; however, were it to be applied, it might only serve to change the direction of antigenic drift. Such a response was recently reported in domestic poultry in Mexico, where vaccination resulted in epidemic levels of a low-frequency strain that would otherwise probably have drifted to extinction [72].

Human-adapted influenza is transmitted rapidly, sometimes even before symptoms are apparent. Recent pandemic strains behaved similarly. We can only guess at the transmission characteristics of future pandemic strains. But based on past observations, isolation of infected patients, which effectively stopped the SARS epidemic [1], will not be an effective option for the control of pandemic influenza.

Epidemiological modeling provides the basis for pandemic planning. Using models, vaccines and antiviral treatment protocols can be evaluated under a wide variety of conditions [41,50,79,99]. Planning ahead is always a good idea. However, if would be nice if we actually had access to the vaccines and antivirals utilized in these models!

Because of technical difficulties arising from aspects of viral replication and host range, as well as biosafety and regulatory issues, the first H5N1 vaccine trial in humans is just now underway [153]. Plasmid-based reversed genetics were used to construct recombinant viruses lacking the string of basic residues in the H5N1 HA cleavage site. Vaccination with these viruses has been effective against challenge with H5N1 viruses in mice [77]. We will not know until clinical trials conclude whether an H5N1 vaccine will become available in the near future. Recent surveillance of birds in China suggests that H5N1 strains are continuing to reassort with other avian viruses [141]. Hopefully, viral evolution will not outpace vaccine development.

Vaccination was recently recommended for control of H5N1 influenza in poultry in Asia. This recommendation came only after culling had failed. Poultry vaccination is controversial in part because poor-quality vaccine can result in apparently healthy birds that nonetheless shed virus [143]. Another argument against vaccination is difficulty in differentiating between vaccinated and infected birds. A strategy proposed to circumvent this is the use of vaccines constructed with the HA of the infecting virus but with a different NA, a mismatch that could be detected serologically [16,73]. Vaccination of poultry with vaccines of questionable quality has already proceeded informally in areas lacking strict government supervision. We will probably never know the extent to which these efforts have contributed to, rather than helped, the problem.

Two classes of antiviral drugs exist for the prophylaxis and treatment of influenza. The indiscriminate dosing of poultry with one group, the adamantadines, has already caused resistance to evolve in H5N1 avian strains in China. Use of the other antiviral drug option, the NA inhibitors, is unlikely to limit or stop a pandemic, firstly because we lack adequate production capacity and stockpiles of these drugs, and secondly, because they would be prohibitively expensive for, and thus unavailable to, most of the people on earth [71, 85,95].

Thus, for all we have learned about influenza evolution, we are essentially just watching, albeit quite attentively, to see whether avian H5N1 influenza will evolve into the next human pandemic strain. It may not. If it does, only a very few humans, those lucky enough to have access to the right drugs, will be protected unless an effective vaccine becomes available in the meantime. However, even the NA inhibitors may only provide short-term protection. Resistance to these drugs will undoubtedly evolve in due time. The influenza virus has already shown us its incredible evolutionary flexibility. There is no predicting what it will do in the future.

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CHAPTER 14

Experimental Evolution of Pathogens

Vaughn S. Cooper

Departments of Microbiology and Genetics, University of New Hampshire, Durham, NH 03820, USA

Why are certain microbes predisposed to causing disease, while others are apparently incapable of this shift? How fast, and by what genetic mechanisms, does this transition to pathogenesis occur? Is the evolution of greater or lesser virulence reversible? Could we have predicted the emergence of a coronavirus capable of causing SARS (Chapter 9, this volume)?

Modern biology typically probes these questions in a stepwise, "bottom–up" fashion: one hypothesizes that an interesting phenotype Y of pathogen A is associated with candidate genetic locus X, the locus is mutated, which affects some part of the phenotype, and then mutant and wild-type strains are evaluated in a well-defined model environment. If results from this analysis are as expected, we typically conclude that *gene* X, *which produces phenotype* Y, *contributes to the virulence of organism A*. We also usually infer that organism A is more virulent than other organisms *because* it has somehow acquired gene X, perhaps by horizontal gene transfer or by mutation of preexisting genes. We then conclude that the predisposition of organism A towards pathogenicity results from gaining gene X and similar elements.

But why organism A in the first place, and not other organisms? And why gene X, but not other loci? Although the bottom–up approach is exceptionally useful up to this point, here it fails. Understanding the origin and predicting the future response of incipient pathogens requires a different strategy. One method, comparative genomics, attempts to associate various microbial lifestyles with the presence or absence of large clusters of genes. This complex, multivariate analysis extends the one-gene, one-phenotype approach and could help predict future evolutionary potential. Another method, experimental evolution, allows the investigator to continuously monitor the genetic and phenotypic response of microbial populations to a controlled laboratory environment. Quite literally, this is "evolution in action," in which the timing, effects, and interdependence of each underlying genetic change can be observed.

Those interested in the evolution of pathogen virulence, for example, might choose a susceptible host model or some other aspect of the pathogenic environment in which to perform experimental evolution. Although each laboratory environment likely favors its own set of adaptive genetic variants, it remains the environment itself and not some heavy-handed manipulation by the investigator that performs the selection. Experimental evolution can therefore be considered a top–down approach that is less biased than a bottom–up, locus-specific manipulation, and better reflects the natural process of genetic adaptation to a new environment or host.

Laboratory evolution experiments involving microbial pathogens (or incipient pathogens) have typically focused on one or more of the following questions: (i) How rapidly will a population of a given microbe evolve increased or reduced virulence? (ii) What are the consequences of this transition to pathogenicity? (iii) What factors promote or retard the emergence of new, more fit or virulent genotypes? (iv) What genetic changes underlie the changes in virulence, and do such changes occur consistently across replicate populations? These four topics are by no means exhaustive, and tend to exclude the complication of introducing foreign genetic material, but represent a cross section of current research.

This chapter focuses on three outstanding, representative examples of the growing number of studies involving experimental evolution of pathogens: one using a virus, one using a bacterium, and one using a primitive eukaryote, yeast. Each example explores multiple factors that could affect the population biology of pathogens and ultimately favor certain genotypes over others in direct competition. More important, these studies describe fundamental properties of microbial evolution that likely extend well beyond the particular organism or infectious process.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

14.1 EXPERIMENTAL DESIGN

In addition to the unique opportunity to study evolutionary processes as they happen, experimental evolution is also appealing because of the significant control it affords the experimenter over both the outcome and the means of analysis. For example, evolutionary pathways can effectively be "replayed" by replicating initially isogenic populations under identical conditions, in effect, "rewinding life's tape" [12]. Even better, given the speed with which microbial populations change relative to human schedules, intermediate steps of evolving lineages can be frozen and reconstituted for analysis at a later date. This "frozen fossil record" [17] permits ancestors and derived lineages to be compared head-to-head without relying on some indirect measure. Finally, genetic markers can be inserted that allow precise counts of different competitors as well as identification or exclusion contaminants from the long-term process.

Nearly all studies begin with a genetically homogeneous bacterial or viral population founded by only a few cells. All subsequent evolutionary change therefore depends on new variation arising within the population. This mutational variation is essential because it provides the grist for the evolutionary mill, so the experimental design must preserve at least a portion of this variation as each population is propagated. Each of the experiments presented below explicitly considers the effect of the "bottlenecking" that occurred during each transfer on the subsequent evolution. The stochastic effects of mutation and population bottlenecks also ensures that each replicate population is an independent evolutionary lineage almost certainly unlike any other. As a result, any commonality, or convergence, among the changes arising in replicate populations is worth noting and can be strong evidence of adaptation to the new environment [2,3,14].

Because during experimental evolution the environment itself is the agent of selection, choice of culture conditions is critical. These can range from liquid broth media, to tissue culture, to complex continuous passage in susceptible animals (Fig. 14.1). Though automated culture devices such as chemostats have been used with great success [9], most experiments employ manual serial batch transfer, in which an aliquot of each culture is introduced into a fresh, uninfected environment on a regular basis. Transfer typically occurs once the population has exhausted the resources of the current environment, which allows the population to enter a period of slow growth or stasis, called stationary phase in bacteria. Once introduced into new medium, the organisms then undergo a delay before resuming exponential growth, called lag phase for bacteria. Despite these varying growth conditions during serial passage, maximum growth rate is often the trait under the strongest selection, as opposed to performance during lag phase or stationary phase [28]. (More complicated culture methods, such as chemostats or turbidostats, may select more strongly on different traits such as resource affinity, or growth efficiency, because in these devices reproduction never ceases.)

14.2 MEASURING ADAPTATION

One of the best ways to evaluate the products of experimental evolution is by competing samples of the evolved populations against their ancestor(s). If the competition environment mimics that of the evolution experiment, we expect the evolved isolates to prevail. But how one exactly quantifies the margin of improvement can be a challenge. Each of the studies presented below applies a different method, each with its own merit.

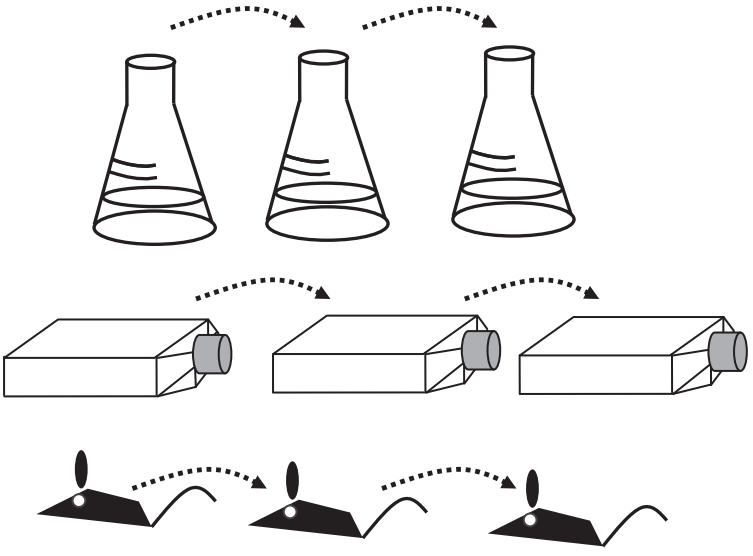

Fig. 14.1. Experimental evolution strategies: serial culture in broth (top); continuous tissue culture (middle); or serial culture in laboratory animals (bottom). Examples of each are presented herein.

The simplest method involves measuring the growth rate of each genotype separately, and then either subtracting the ancestral value to yield a difference, or dividing by the ancestral value to produce a ratio. This frequently provides a sufficient picture, but sometimes adaptation is only detectable in the presence of competitors, which could be overlooked by this technique. For example, one genotype may evolve the ability to antagonize other competing genotypes by secreting a toxin or sequestering a key nutrient, and such effects would only be visible in head-to-head competition.

In direct competition, however, a marker must be introduced to distinguish the ancestor from the evolved isolates. Unfortunately, markers usually affect the growth of the marked genotype and usually require enumeration on different plates (e.g., one containing and one lacking an antibiotic), which introduces an extra measure of experimental variance. The best markers, on the contrary, affect fitness in few if any environments and can be visualized in both marker states on the same plate. Given one of these markers, evolved and ancestral genotypes of different markers can be introduced into the same environment, counted at time $= 0$, and then counted again at a later time. Here, the genotype that undergoes greater reproduction during the competitive interval is by definition the one with greater *fitness*. The key parameter, relative fitness, can be calculated as the ratio of the realized growth rates (doublings) of the two competitors using the following equation:

$$W_{ij} = \frac{\ln[N_i(1) / N_i(0)]}{\ln[N_j(1) / N_j(0)]}$$

Here the fitness of strain i relative to strain j, W_{ij}, is the ratio of the genotype densities, N_i and N_j, at times 0 and 1 day. A slightly different fitness measure, the *selection rate constant*, can also be calculated:

$$s = \frac{\ln[N_i(t) / N_i(0)] - \ln[N_j(t) / N_j(0)]}{t}$$

Here, the selection rate constant, s, is the *difference* in the rates of growth (per unit time), whereas relative fitness W is the corresponding *ratio*. Because fitness depends on the ratio of rates, it is dimensionless and thus may be more readily compared across different environmental or genetic contexts.

Another way to analyze the outcome of head-to-head competition is by plotting the ratio of the two competitors over time and then determining the slope of this function over time from a linear regression. This method is especially useful when only the ratio of the two competitors, and not the actual number of total individual genotypes, can be calculated; this is common in experiments using viruses that grow in inherently variable environments such as tissue culture. Fortunately, given a data set with known starting and final numbers and frequencies, we can calculate relative fitness, selective rate constant, and fitness based on linear regression simultaneously (Fig. 14.2), and choose the best of these for our experiment.

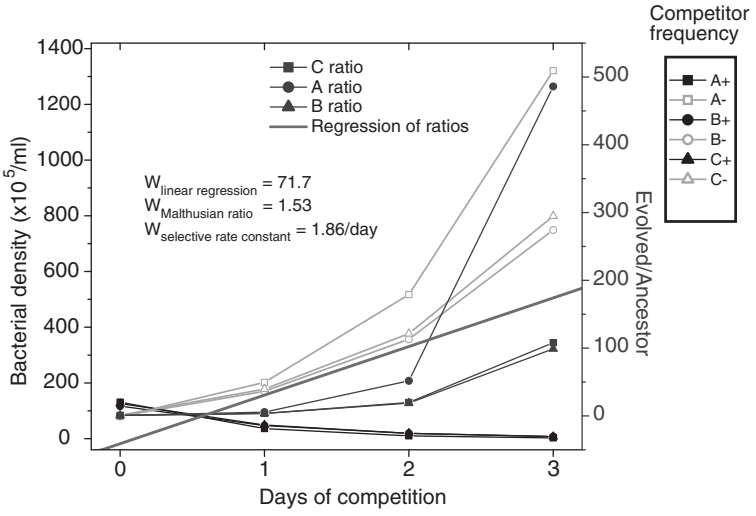

Fig. 14.2. Competition between evolved and ancestral genotypes derived from a 20,000 generation evolution experiment involving *E. coli* growing in a simple salts medium with glucose as the sole carbon source [3,16,17]. Relative frequencies of the ancestor and evolved genotypes are plotted as raw counts (left ordinate) and as ratios (right ordinate) over time. Fitness of the evolved genotypes is calculated relative to the ancestor by each of the three different methods described in the text. W = fitness. See color plates.

14.3 EXPERIMENTAL EVOLUTION OF VESICULAR STOMATITIS VIRUS (VSV)

One of the best studied models of viral evolution uses vesicular stomatitis virus (VSV), a negative-sense RNA virus with a small genome (11.2 kb) that is capable of infecting a broad range of insect vectors and vertebrate hosts [19,23]. VSV is typically cultured in one of a few well-defined tissue culture systems. One cell type is baby hamster kidney cells (BHK-21), which serves as an analog of a mammalian host; another is derived from the sand fly (LL-5) and mimics the vector host. VSV researchers benefit from a well-defined marker, resistance to a monoclonal antibody (MARM), which does not alter the outcome of direct competition with an unmarked relative. Changes in various populations of evolving VSV can therefore later be compared relative to this marked progenitor.

When VSV populations have been serially transferred in a single tissue culture system, astounding rates of adaptation to that environment have been frequently observed [19,23]. In some cases, relative fitness may approach values five- or even 10-fold higher than the ancestor, which is a considerably greater margin than found in other systems [19]. One unresolved problem is whether viral adaptation to a particular cell type is specific to that environment, or more precisely, whether adaptation to a certain cell type either enhances or compromises growth in different cell types. Because one underlying assumption of vaccine design is that prolonged culture in a foreign environment will attenuate virulence in susceptible hosts, controlled studies of this process are especially useful. One potential outcome is that selection in a foreign environment actually enhances fitness in the susceptible host environment (Fig. 14.3A). This correlated adaptation may actually reflect adaptation to growth in the laboratory in general, and not a certain cell type in particular. Another possibility is that fitness in the original host is unaffected by adaptation to the new cell type (Fig. 14.3B). A final, perhaps more desired outcome from the perspective of the vaccine designer is reduced fitness in the susceptible host environment, caused by a genetic trade-off (Fig. 14.3C).

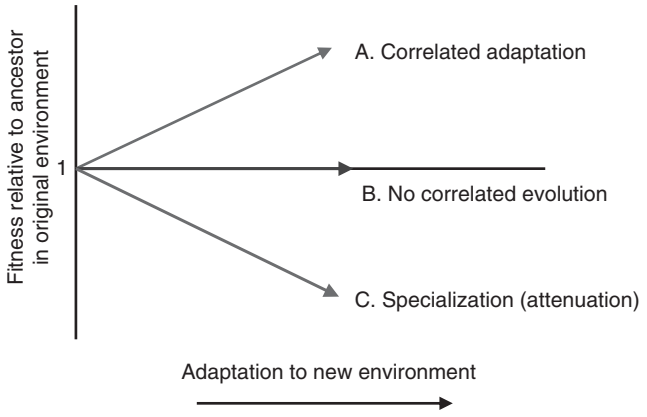

Fig. 14.3. Hypothetical outcomes of adaptation to a new environment or host when fitness is quantified in the original environment or host. (A) correlated adaptation; (B) no correlated change; (C) evolutionary specialization or attenuation.

Trade-offs may be caused by either of two mechanisms: antagonistic pleiotropy or mutation accumulation. Under antagonistic pleiotropy, mutations that enhance fitness in the selected environment (cell type) also reduce fitness in other environments (cell types); thus, specialization is caused directly by selection. Under mutation accumulation, however, different sets of mutations improve performance in the selected environment and reduce performance elsewhere. The underlying cause is the genetic drift and ultimate fixation of mutations that harm traits no longer under selection in the favored environment; thus, loss of fitness in alternative environments results from an absence of selection. In theory, specialization by mutation accumulation may yield a more stably attenuated vaccine candidate, because such evolution may involve multiple mutational steps and prove less reversible, unlike the forward-and-back see-saw of single pleiotropic mutations.

Several groups have explored the potential for VSV to adapt to specific tissues and encumber trade-offs in other environments. Novella et al. [22] demonstrated one extreme example of a trade-off by culturing VSV populations for 10 consecutive months in sand fly cells, and then evaluating their competitive fitness in two mammalian cell environments (BHK-21 tissue culture and mouse brains). They found that sand fly-evolved VSV were at least 10 times as fit as the ancestor in sand fly cells, but the ancestral virus was at least 10-fold as fit as sand fly-evolved viruses in both mammalian environments (represented by Fig. 14.3C). Surprisingly, this clear trade-off did not persist once the sand fly-evolved VSV were passaged only once at high density in BHK-21 tissue culture, which restored virulence in both mammalian environments. Clearly, this process of attenuation was unstable and alarmingly reversible, which does not bode well for certain vaccine design strategies.

Turner and Elena [27] also demonstrated that adaptation to new cell types by VSV was typically associated with a loss of fitness on BHK cells. Specifically, populations evolved on human epithelia carcinoma (HeLa) cells and those alternating between HeLa and Madin-Darby canine kidney (MDCK) were, on average, only one-quarter as fit as the ancestor on BHK cells, but anywhere from two- to eight-fold more fit than the ancestor in their selective environment (as in Fig. 14.3C). However, populations evolved in MDCK cells alone were typically more fit (1.4–3.8-fold) than the ancestor on BHK cells, an example of correlated adaptation to an unseen environment (Fig. 14.3A). Adaptation by VSV to specific tissues may therefore either result in reduced fitness or increased fitness in alternative environments, and these alternatives are difficult to predict lacking previous experimentation.

Given the obvious adaptive potential of VSV populations, Zarate and Novella [29] asked why natural populations of

VSV are evidently stable from year to year, varying only between distinct ecotypes. They hypothesized that the VSV life cycle alternating between insect vector and vertebrate constrains the evolution of viral populations and favors stable intermediate phenotypes capable of growth in both hosts. Because Turner and Elena [27] had previously shown that acute infection in alternating cell types promoted adaptation to both environments, Zarate and Novella [29] changed the infection dynamic and studied the more realistic effect of persistent (non-acute) infections in sand fly cells alternating with acute infections in BHK cells. Thus, they established three sets of VSV lineages: (i) alternating between persistent replication in LL-5 sand fly cells and acute infection in mammalian BHK cells, (ii) continuous persistent infection in LL-5 cells, and (iii) repeated acute infections in BHK cells. Fitness was assessed by calculating the slope from the ratio of the two competitors over time from a linear regression.

First, Zarate and Novella [29] found that viruses replicating in alternating environments and those replicating solely in LL-5 cells were several-fold better than the ancestor in LL-5 cells, but significantly worse on BHK cells (Fig. 14.4). Only repeated acute infections in BHK led to significant adaptation to that cell type, and any persistent infection in LL1 cells compromised adaptation to BHK cells. We can therefore infer that persistent infection in insect cells, and not the mammalian environment, clearly dominates the selective forces acting on VSV; otherwise, populations experiencing alternating environments would have adapted to both cell types. This implies that acute infections of vertebrates may be less relevant to the evolution of the larger VSV population, though these infections certainly serve to amplify viral populations to huge numbers.

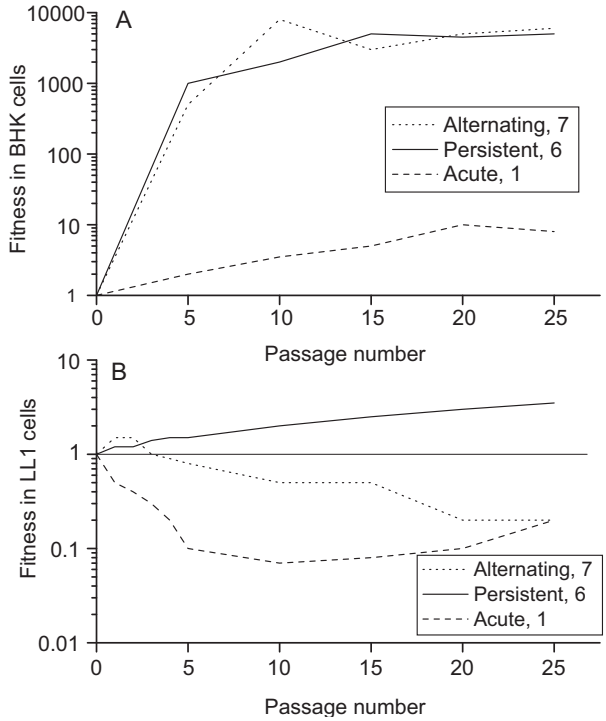

Fig. 14.4. Fitness of VSV populations assayed in (A) mammalian cells (BHK) or (B) insect cells (LL1). Populations were propagated as persistent infections of insect cell lines (persistent), as acute infections of mammalian cells (acute), or alternating between the two cell types (alternating). Total number of genetic substitutions identified in each population is displayed under each trajectory. Adapted from Zarate and Novella [29].

Second, to better characterize the molecular mechanisms underlying this assymetrical trade-off, Zarate and Novella sequenced the entire RNA genome of selected evolved and ancestral isolates from the experiment. They observed only one replacement substitution in the isolate from the BHK-restricted population, whereas they found six replacement substitutions in the LL1-restricted population. Interestingly, those populations alternating between the two environments acquired nearly the same set of substitutions as the LL1-restricted population, and most of these mutations became common early in the course of the 25-passage experiment. The lack of substantial genetic change in the final 10 passages coincided with a lack of further adaptation during this interval. Thus, despite the long history of passaging VSV in a variety of mammalian cell types and observing drastic rates of adaptation, it appears that in the wild, VSV evolution is dominated by long-term chronic infection of insect populations, whose environment constrains viral populations to a relatively narrow range of genotypes. Such knowledge of the selective forces that act on viral populations can certainly inform future vaccine design by illustrating those strategies that should be most efficient in producing attenuated, host-specialized genotypes.

14.4 IN VIVO EVOLUTION OF *SALMONELLA TYPHIMURIUM*

A wide variety of estimates exist for rates of bacterial adaptation *in vitro*, but far less exist for *in vivo* conditions. To fill this gap, and to quantify the effects of mutation rate and the size of the transferred population on adaptation, Nilsson et al. [20] serially passaged 18 independent lineages of *Salmonella typhimurium* LT2 in BALB/C mice by intraperioteneal injection and subsequent recovery of the infected spleen. The 18 lineages comprised four groups: (i) wild-type mutation rate (5×10^{-8} for nalidixic acid resistance) and low population size (10^3), (ii) wild-type mutation rate and high population size (10^5), (iii) 700-fold increased mutation rate ($\sim 7 \times 10^{-5}$) and low population size, and (iv) increased mutation rate and high population size. Both theory [8,10,26] and empirical evidence [8,11,13] suggest that these two experimental variables, mutation rate (μ) and effective population size (N_e), are key determinants of the rate of microbial adaptation to a new environment, but that neither variable can predict this rate alone. Together, mutation rate and population size determine the supply rate of beneficial mutations – the lower μ or N_e, the fewer favorable mutations arise in each population, and hence the slower adaptation proceeds.

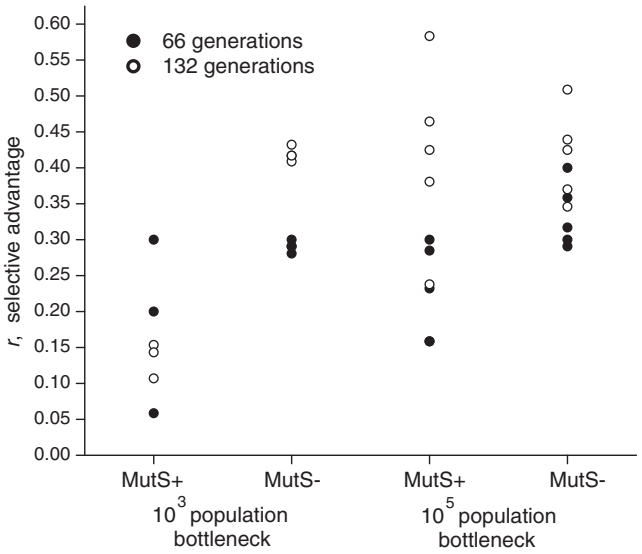

Fig. 14.5. Fitness of *Salmonella typhimurium* populations following 66 or 132 generations of growth in serial murine infections. Populations were either MutS proficient (wild type) or deficient (mutator) and experienced population bottlenecks between infections of 10^3 or 10^5 cells. Fitness was quantified as the selective rate constant expressed relative to the ancestor; see text for details. Adapted from Nilsson et al. [20].

After fewer than 200 generations of adaptation to the endothelial cell environment of the mouse, Nilsson et al. [20] observed selective coefficients, or relative advantages, ranging from 0.11 to 0.58 per generation. Adaptation occurred more quickly for the lineages with higher mutation rates at both population sizes, but lineages with lower mutation rates eventually narrowed the gap in relative fitness in later generations. The small N_e, low μ group was the least well adapted, whereas the small N_e, high μ group was nearly as fit as those groups passaged with higher N_e (Fig. 14.5). These results are consistent with the findings of de Visser et al. [8], who observed that N_e and μ combine to set a "speed limit" on adaptation, which results from a saturation of the number of competing favorable mutations. These findings clearly apply to pathogens that undergo population bottlenecks during the infectious process. During such bottlenecks, genetic variation among individuals will be low, so increased genomic mutation rate may provide a short-term advantage to the population by generating more adaptive variation that may help elude host defenses.

However, increased mutation rate may also be costly to a microbial population. Because most mutations are deleterious, populations with an elevated mutation rate generate more unviable offspring as well as a much larger pool of less fit individuals. Most of these unfit offspring are weeded out by natural selection, but "mutator" populations also accumulate mutations in unused traits by the persistent force of genetic drift, thus accelerating the evolution of specialization. To test the effect of increased mutation rate on mouse-adapted populations of *S. typhimurium*, Nilsson et al. [20] quantified the metabolic capacity and frequency of auxotrophy in each population. They also quantified fitness relative to the ancestor in simple broth at three temperatures, predicting that *in vivo* selection would compromise *in vitro* growth. Somewhat surprisingly, they found no overall loss of fitness under laboratory conditions, and some non-mutator lineages even increased their ability to grow in broth. On the contrary, all mutator lineages harbored a variety of auxotrophs, ranging from 0.7% to 39% of the total population, whereas auxotrophs were undetected in non-mutator populations. Further, all of the mutator lineages had lost at least one of the metabolic functions screened, whereas none of the wild-type lineages acquired any defects. Clearly, increased mutation rate accelerated adaptation to the mouse environment, but this reduced the overall functionality of the mutator populations, which could conceivably compromise their long-term viability outside a particular host.

How exactly did these populations of *S. typhimurium* adapt to colonizing mice by intraperitoneal injection? Research is still ongoing to identify the specific mutations responsible for the ~10–50% improvement in competitive growth, but the authors were able to determine some important components of this adaptive process. Most notably, the pace and magnitude of adaptation was quite high, which provides strong evidence that numerous sites in the genome were capable of producing beneficial alleles. Had only a few nucleotide substitutions produced favorable variation, the rate of adaptation would have been much slower and the variation among replicates of each group likely would have been higher, owing to the stochastic appearance of these few rare mutations. Rather, Nilsson et al. estimated that 10^4 bp of the *S. typhimurium* genome or more could generate favorable variation in this system, which may reflect the complexity of the mouse environment. The course of infection involves uptake by macrophages at peripheral lymph nodes and then transport by blood and lymph to the liver and spleen, and it is likely that a variety of substitutions may increase the efficiency of each of these steps [20]. It is no wonder, then, that potential but not obligate pathogens such as *S. typhimurium* can cause successful infections from relatively few founders originating from a variety of environments [24]: even maladapted genotypes may rapidly become more efficient because of a wide variety of potential genetic adaptations.

14.5 EXPERIMENTAL EVOLUTION OF *CANDIDA ALBICANS* ANTIBIOTIC RESISTANCE

Relatively few experimental evolution projects have been performed with microbial eukaryotes (Zeyl et al. [13,30] and Luckinbill [18] are two of several notable exceptions), let alone microbial eukaryotic pathogens. Yet some microbial eukaryotes are among the world's worst pathogens and thus further research into the factors that govern their evolution is essential. One series of studies by Cowen et al. [5–7] tackled this challenge by following adaptation by the pathogenic fungus

Candida albicans to inhibitory concentrations of the antimicrobial drug fluconazole. Six independent populations were exposed to either steady or increasing concentrations of the antibiotic, depending on the level of the most recently measured minimum inhibitory concentration (MIC). Six additional populations were passaged in the absence of antibiotic; these served as controls for adaptation to the laboratory environment in general and not the antibiotic. As in the previous studies, each of these populations was founded by a single clone with no capacity for genetic exchange between individuals, so all variation arose *de novo* by mutation during the experiment. This stochastic variation arising uniquely within each population allowed the authors to study how chance affects the evolution of azole resistance in *C. albicans*.

The authors predicted three possible genetic and phenotypic outcomes (Fig. 14.6): (A) all populations could adapt to the experimental environment by mutations in the same few loci and evolve identical phenotypes, which implies that only one adaptive solution to fluconazole exists for *C. albicans*; (B) each population could acquire one of a few different combinations of mutations, which implies that adaptation is constrained to a limited number of pathways; (C) each population could acquire its own unique combination of mutations with separate consequences, which implies that adaptation may proceed along many different pathways.

The initial findings were somewhat surprising and seemed to favor the last of these outcomes (Fig. 14.6C). Each population evolved different levels of drug resistance over distinctly different trajectories, which was accompanied by different growth rates in the absence of drug and different expression patterns in four genes known to be associated with azole resistance (Fig. 14.7A). In addition, the authors found genetically distinct lineages within the populations that were

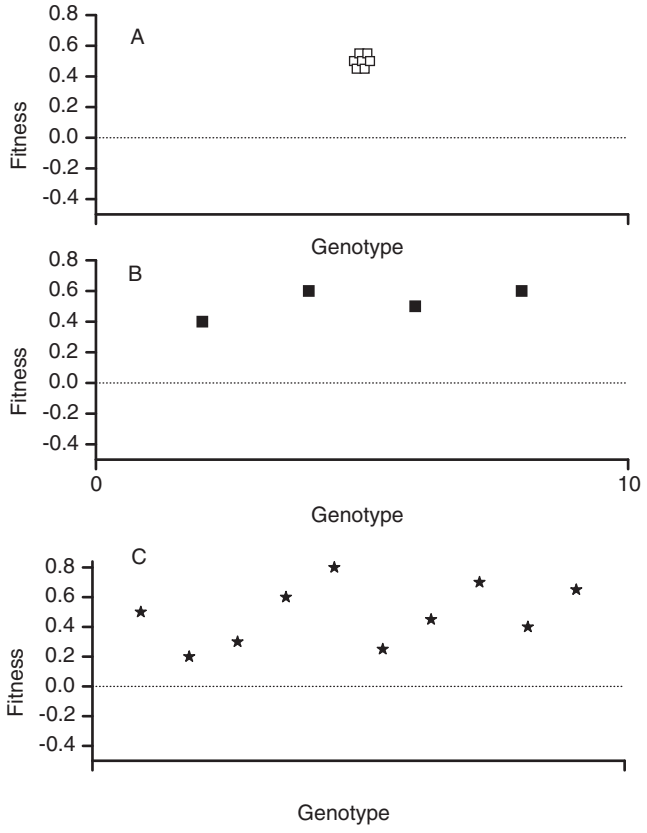

Fig. 14.6. Alternative evolutionary outcomes of replicate populations adapting to a common, novel environment. Fitness is expressed relative to the ancestor = 0 (dotted line). (A) Only one genetic solution exists to the challenges posed by the new environment, so all populations achieve the same level of fitness by the same mechanism. (B) Replicate populations converge upon relatively few genotypes that permit adaptation to the new environment, with some variation in relative fitness. (C) Each replicate population adapts to the new environment by different genetic combinations that produce different levels of relative fitness.

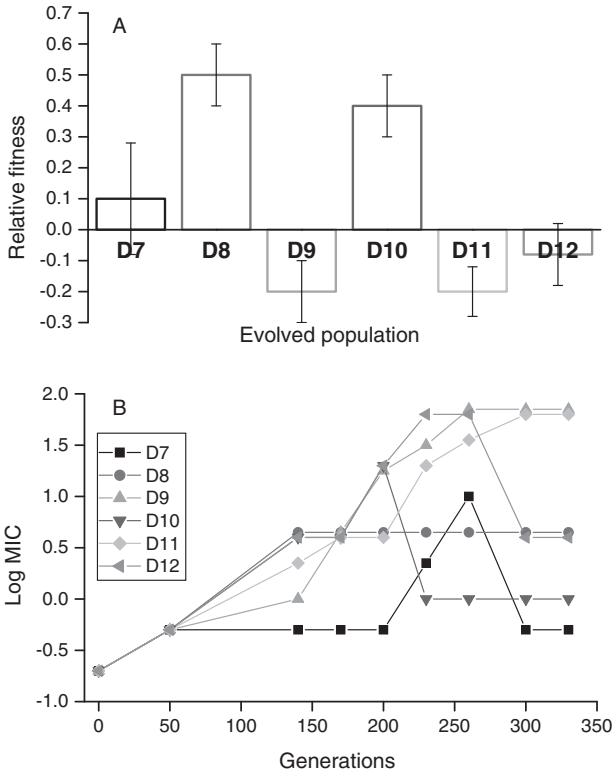

Fig. 14.7. Relative fitness (A) and evolved level of fluconazole resistance (B) of six populations of *C. albicans* propagated for 330 generations in the presence of increasing levels of the antimicrobial drug fluconazole. Here, fitness was quantified in a medium lacking fluconazole and estimated as the difference in the numbers of doublings between the evolved population and the genetically marked progenitor, standardized by the total number of doublings in the assay, which varied across environments. The six populations achieved approximately three different levels of fitness relative to the ancestor. Some of the variation within populations in fluconazole resistance over time resulted from fluctuations in different genotypes that coexisted in the experimental environment. Note that the less fit populations are among the most resistant to fluconazole. From Cowen et al. [6]. See color plates.

maintained over time, rather than sequentially excluding the preceding lineage. When they analyzed several neutral genetic markers that were heterozygous in the ancestor, they found that some populations became homozygous at an intermediate sample, but then later reverted to the ancestral heterozygosity. Because the probability that two lineages differing in their genotype at this locus coexisted in the population is much greater than a forward- and back-mutation at the same site, they concluded that multiple competing genotypes persisted throughout the experiment. Thus, the evolution of azole resistance in *C. albicans* populations may involve variable combinations of distinct genotypes, rather than a predictable succession of mutational events in a simple lineage.

However, when Cowen et al. [6] quantified the fitness of the six *C. albicans* populations evolved in the presence of fluconazole in an environment lacking antibiotic (Fig. 14.7B), they determined that the populations probably fell into three different groups with different levels of fitness. The number of genetic solutions to the experimental environment containing fluconazole was therefore apparently limited. The authors dug further into the genetic architecture of the evolved fluconazole resistance in *C. albicans* by performing expression microarray analysis on four of the six replicate evolved populations as well as the ancestral strain [7]. They identified 301 of more than 5000 ORFs with significantly altered expression, but these were reduced to only three distinct patterns of genetic adaptation over time. One pattern was unique to one population and involved upregulation of a known multidrug ATP-binding cassette transporter gene, CDR2. The other two patterns were found as successive steps in the other populations, one that occurred early in two populations, and another that occurred late in all three remaining populations. Both of these patterns were complexes of expressed changes that upregulated a multidrug major facilitator transporter, MDR1, which seems to represent a dominant program for the evolution of azole resistance. Even more notable was their finding that many fluconazole-resistant clinical isolates of *C. albicans* also express the same three suites of genetic expression. Thus, a clearer interpretation of this adaptive landscape, a metaphor describing the process by which different populations climb to levels of greater fitness, is that it is dominated by only a few peaks, or adaptive solutions, that all populations converge upon.

One hope in spite of the widespread use of antibiotics and the growing frequency of resistant pathogens is that antimicrobial resistance is typically costly to the resistant individual in environments lacking the antibiotic compound. This trend is borne out in a wide range of prokaryotic and eukaryotic microbes, though the magnitude of the cost varies substantially [1]. However, it is possible that *prolonged* antibiotic use could reinforce the stability of resistance in the absence of antibiotic by selection for alleles that compensate for any incurred cost. Cowen et al. [5,7] observed varying levels of fluconazole resistance among the populations evolving in the presence of this antibiotic, and also significantly more variation among these replicates than among populations evolved without antibiotic. Apparently, the evolutionary trajectories of *C. albicans* populations become less predictable in simple culture once antibiotic is added. One notable pattern was a transient increase, and then a *reduction* in the MIC of fluconazole for several populations that evolved under increasing antibiotic concentrations (Fig. 14.7B). The first increase in MIC was typically accompanied by a cost in fitness relative to the sensitive ancestor, but the subsequent reduced MIC eliminated that cost. Even more intriguing was their finding that antibiotic-evolved populations could grow at fluconazole concentrations well beyond their measured MIC, which means that the selected genotypes had eluded an apparent trade-off between level of resistance and competitive ability. Such genotypes may underlie the clinical problem of failure to treat cases of *C. albicans* infection in which the genotype was originally typed as "sensitive" by standard means.

The finding that multiple genetic solutions exist for microbes confronted with antimicrobial compounds is certainly troublesome because it affects the design of public health strategies to manage the emergence and spread of resistance. Should most genetic causes of resistance cause that microbe to grow less efficiently than susceptible relatives in the absence of antibiotic, then resistant genotypes will wane and ultimately disappear from the population. However, other genotypes that balance resistance with overall growth efficiency have been shown to evolve *in vitro* [1,15,25], which makes the preservation of antibiotic susceptibility among pathogens a challenge indeed. It is presently unclear whether resistant genotypes can be formidable competitors in their natural environment, outside of test tubes or even the highly favorable setting of hospitals doused in antibiotics. Antimicrobial resistance may also interfere with the ability of the pathogen to compete within the larger natural microbial community, a dynamic that should be tested in more realistic microcosm experiments.

14.6 FUTURE PROSPECTS

What have these three examples of experimental evolution of pathogens taught us? First, following the evolution of pathogenic microbes in the laboratory allows us to quantify their rate of adaptation to challenging environments or hosts. Some organisms are evidently extremely capable of rapid adaptation to certain environments, such as VSV in BHK cells, whereas others are far more constrained, such as VSV in sand fly cells [29]. Second, replicate populations founded by the same starting genotype often evolve different solutions to the same environment as a result of alternative favorable mutations arising by chance. This variation among replicates was observed in all three examples presented here – in a virus, in a bacterium, and in yeast [6,20,29]. Thus, chance can have a considerable effect on the evolutionary outcome of pathogens introduced into a new environment. Further, the alternative genetic strategies may vary in their clinical significance or manifestation; for example, the variability among the *C. albicans* populations evolved in the presence of

antibiotic produced traits that resist treatment in infected patients. Third, advances in genome-wide screening techniques have made it possible to pinpoint each relevant substitution in evolving pathogenic populations, which was formerly far more laborious and uncertain. It is now possible to trace changes in populations of pathogenic microbes, from the coarsest phenotype to the most precise genetic consequence, within reasonable human time frames. Lastly, they illustrate the potential of experimental evolution as a method to better understand the biology of extant pathogens and new emerging infectious diseases. Properties of the evolution of many microbes can be studied in defined laboratory environments, which enables the pursuit of any of the following questions, each of which remains largely untested.

(i) Are genes known to affect pathogen virulence, especially those that were evidently acquired recently from other organisms, selectively *optimal* in their various environments and *stable* in their current host? Do some environments make the possession or expression of these virulence traits deleterious, which could guide new strategies to combat the disease agent?
(ii) Is the evolutionary transition to pathogenicity typically accompanied by specialization that compromises the ability to persist and grow in alternative environments? Evidence is growing that long-term selection in a given environment will affect the ability to grow elsewhere [3,4,20,21], but is this a general property of emerging pathogens?
(iii) How does the introduction of mechanisms of intergenomic recombination, such as phage, plasmids, and natural DNA transformation, affect the progress and outcome of experimentally evolved pathogens? Are incipient pathogens with greater ability for genetic exchange more adaptable, and thus potentially of greater concern?
(iv) Can closely controlled and monitored populations of experimentally evolving pathogens provide insight into improving the efficiency of vaccine design, perhaps by highlighting fundamental mutational tendencies or consequences?

Given the global resurgence of infectious microorganisms as causes of morbidity and mortality, the need to streamline treatment and vaccine design is obvious, but integrating this industry with the much smaller but rigorous field of experimental evolution could prove extremely valuable. The contributors to this volume will hopefully provide a good start to this synthesis.

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GLOSSARY

Adaptive landscape: A metaphor or three-dimensional image that describes the relative *fitness* of combinations of different sets of gene variants (alleles), some being of low value and producing valleys, and others being of high value and producing peaks.

Allele: Any particular gene variant.

Attenuation: The process by which pathogens are weakened in *pathogenicity* or *virulence*, especially for vaccine construction.

Ecotype: A species subset that persists as a distinct group as a result of specific environmental selection and isolation.

Fitness: The capability of an individual of certain *genotype* to reproduce, which typically reflects the proportion of the individual's *alleles* among all *alleles* present in the next generation.

Genotype: The totality of gene variants that describe an individual organism.

Genomics: The study of all of the nucleotide sequences of the chromosomes of an organism, as well as their organization.

Isogenic: Of exactly the same *genotype.*

Pathogenicity: The capacity to cause disease.

Phenotype: The visible or quantifiable properties of an organism that are produced by the interaction of the genotype with the environment.

Stochastic: Random, especially in a mathematical sense.

Virulence: The degree to which a parasite reduces the fitness or function of its host.

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CHAPTER 15

Evolution of Antigenic Variation[1]

Steven A. Frank

Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697–2525, USA

15.1 INTRODUCTION

Infectious disease remains a major cause of morbidity and mortality. Consequently, great research effort has been devoted to parasites and to host immune responses that fight parasites.[2] This has led to rapid progress in understanding the biology of parasites, including the molecular details about how parasites invade hosts and escape host immune defenses. Vaccines have followed, sometimes with spectacular success [45].

But many parasites escape host defense by varying their *antigenic* molecules recognized by host immunity. Put another way, rapid evolution of antigenic molecules all too often prevents control of parasite populations. The challenge has been to link molecular understanding of parasite molecules to their evolutionary change and to the antigenic variation in populations of parasites.

The growth of information about antigenic variation provides a special opportunity. For example, one can find in the literature details about how single amino acid changes in parasite molecules allow escape from antibody binding, and how that escape promotes the spread of variant parasites. Evolutionary studies no longer depend on abstractions – one can pinpoint the physical basis for success or failure and the consequences for change in populations.

Molecular understanding of host–parasite recognition leads to a comparative question about the forces that shape variability. Why do some viruses escape host immunity by varying so rapidly over a few years, whereas other viruses hardly change their antigens? The answer leads to the processes that shape genetic variability and evolutionary change. The causes of variability and change provide the basis for understanding why simple vaccines work well against some viruses, whereas complex vaccine strategies achieve only limited success against other viruses.

The battle between host and parasite often comes down to the rates at which attacker and defender molecules bind or evade each other. The biochemical details of binding and recognition set the rules of engagement that shape the pace, scale, and pattern of diversity and the nature of evolutionary change [45].

The first section of this chapter lists the different ways in which parasites can gain by varying their antigenic molecules. The parasites may, for example, extend their time of infection within a particular host by changing their antigenic molecules to evade the specific recognition built up by the host immune system. Or the parasites may vary to attack hosts that had been exposed previously and had built up specific recognition against prior antigenic molecules.

The second section focuses on the nature of binding and recognition between host and parasite molecules. I summarize the different ways in which parasites generate new variants in order to escape molecular recognition.

The third section builds up the individual molecular interactions into the dynamics of a single infection within a host. The parasites spread in the host, triggering immune attack against dominant antigens. The battle within the host develops through changes in population numbers – the numbers of parasites with particular antigens and the numbers of immune cells that specifically bind to particular antigens.

The fourth and fifth sections discuss how the successes and failures of different parasite antigens within each host determine the rise and fall of parasite variants over space and time. The distribution of parasite variants sets the immune memory profiles of different hosts, which in turn

[1]This chapter is a condensed version of my book, *Immunology and Evolution of Infectious Disease*, Princeton University Press, Princeton, NJ, 2002.

[2]I use the word *parasite* for all infectious agents, including viruses, bacteria, and protozoa.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

shape the landscape in which parasite variants succeed or fail. These coevolutionary processes determine the natural selection of antigenic variants and the course of evolution in the parasite population. I discuss several methods that can be used to infer the evolutionary processes that shape antigenic variation.

15.2 WHY DO PARASITES VARY?

In this section, I describe the benefits that antigenic variation provides to parasites. These benefits help to explain why parasites vary in certain ways.

15.2.1 Extend Length of Infection

Trypanosoma brucei, the protozoan parasite responsible for human African trypanosomiasis (formerly sleeping sickness), changes its dominant antigenic surface glycoprotein at a rate of 10^{-3} to 10^{-2} per cell division [138]. The trypanosome changes to another surface coat by altering expression between different genes already present in the genome. Infections lead to successive waves of parasitemia and clearance as novel antigenic types spread and are then checked by specific immunity.

Some viruses, such as HIV, escape immune attack by mutating their dominant epitopes [86]. Mutational changes to new, successful epitopes may be rare in each replication of the virus. But the very large population size of viruses within a host means that mutations, rare in each replication, often occur at least once in the host in each parasite generation.

For parasites that produce antigenic variants within hosts, the infection continues until the host controls all variants, raises an immune response against a non-varying epitope, or clears the parasite by nonspecific defenses. Antigenic variation can extend the total time before clearance [36,47,89]. Extended infection benefits the parasite by increasing the chances for transmission to new hosts.

15.2.2 Infect Hosts with Prior Exposure

Host immune memory recognizes and mounts a rapid response against previously encountered antigens. Antigenic variants that differ from a host's previous infections escape that host's memory response. The distribution of immune memory profiles between hosts determines the success of each parasite variant.

In the simplest case, each antigenic type acts like a separate parasite that does not cross-react with other variants. As host individuals age, they become infected by and recover from different antigenic variants. Thus, the host population can be classified by resistance profiles based on the past infection and recovery of each individual [7].

Two extreme cases define the range of outcomes. On the one hand, each variant may occasionally spread epidemically through the host population. This leaves a large fraction of the hosts resistant upon recovery, driving that particular variant down in frequency because it has few hosts it can infect. The variant can spread again only after many resistant hosts die and are replaced by young hosts without prior exposure to that antigen. Variants may, on the other hand, be maintained endemically in the host population. This requires a balance between the rate at which infections lead to host death or recovery and the rate at which new susceptible hosts enter the population. The parasite population maintains as many variants as arise and do not cross-react, subject to "birth–death" processes governing the stochastic origin of new variants and the loss of existing variants.

15.2.3 Infect Hosts with Genetically Variable Resistance

Host genotype can influence susceptibility to different parasite variants. For example, MHC genotype determines the host's efficiency in presenting particular epitopes to T cells. From the parasite's point of view, a particular antigenic variant may be able to attack some host genotypes but not others.

Hill [55] pointed out that hepatitis B virus provides a good model for studying the interaction between MHC and parasite epitopes. Preliminary reports found associations between MHC genotype and whether infections were cleared or became persistent [6,57,136]. The hepatitis B virus genome is very small (about 3000 base pairs, or bp), which should allow direct study of how variation in viral epitopes interacts with the host's MHC genotype. Host genotype can also affect the structure of the cellular receptors to which parasites attach. For example, the human CCR5 gene encodes a coreceptor required for HIV-1 to enter macrophages. A 32 bp deletion of this gene occurs at a frequency of 0.1 in European populations. This deletion prevents the virus from entering macrophages [81,96,122]. It is not clear whether minor variants of cellular receptors occur sufficiently frequently to favor widespread matching variation of parasite surface antigens. Several cases of this sort may eventually be found, but in vertebrate hosts genetic variation of cellular receptors may be a relatively minor cause of parasite diversity.

15.2.4 Vary Attachment Characters

Parasite surface antigens often play a role in attachment and entry into host cells or attachment to particular types of host tissue. Varying these attachment characters allows attack of different cell types or adhesion to various tissues. Such variability can provide the parasite with additional resources or protection from host defenses. Protozoan parasites of the genus *Plasmodium* cause malaria in a variety of vertebrate hosts. Several *Plasmodium* species switch antigenic type [22]. Switching has been studied most extensively in *Plasmodium falciparum* [109]. Programmed mechanisms of gene expression choose a single gene from among many archival genetic copies for the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [30]. As its name implies, the parasite expresses this antigen on the surface of infected erythrocytes. PfEMP1 induces an antibody response, which likely plays a role in the host's ability to control infection [109]. PfEMP1 influences

cytoadherence of infected erythrocytes to capillary endothelia [109]. This adherence may help the parasite to avoid clearance in the spleen. Thus, antigenic variants can influence the course of infection by escaping specific recognition and by hiding from host defenses [109]. Full understanding of the forces that have shaped the archival repertoire, switching process, and course of infection requires study of both specific immune recognition and cytoadherence properties of the different antigenic variants.

HIV provides another example. This virus links its surface protein gp120 to two host-cell receptors before it enters the cell [96]. One host-cell receptor, CD4, appears to be required by most HIV variants (but see [117]). The second host-cell receptor can be CCR5 or CXCR4. Macrophages express CCR5. A host that lacks functional CCR5 proteins apparently can avoid infection by HIV, suggesting that the initial invasion requires infection of macrophages. HIV isolates with tropism for CCR5 can be found throughout the infection; this HIV variant is probably the transmissive form that infects new hosts. As an infection proceeds within a host, HIV variants with tropism for CXCR4 emerge [96]. This host-cell receptor occurs on the surface of the $CD4^+$ (helper) T lymphocytes. The emergence of viral variants with tropism for CXCR4 coincides with a drop in $CD4^+$ T cells and onset of the immunosuppression that characterizes AIDS. These examples show that variable surface antigens may sometimes occur because they provide alternative cell or tissue tropisms rather than, or in addition to, escape from immune recognition.

15.3 MECHANISMS THAT GENERATE VARIATION

In this section, I summarize the different ways in which parasites generate antigenic variants. The amount of new variation and the kinds of new variants influence antigenic polymorphism and the pace of evolutionary change [36,47,89].

15.3.1 Mutation and Hypermutation

RNA virus populations typically have high frequencies of mutants and often evolve rapidly. However, few studies have provided direct estimates of mutation rates. The limited data suggest relatively high mutation rates on the order of 10^{-4} to 10^{-5} per base per replication [31,38,39,106].

Drake et al. [38] summarized mutation rates for various microbes with DNA chromosomes. They found an amazingly consistent value of approximately 0.003 mutations per genome per generation. This value holds over genomes that vary in total size by from 6×10^3 to 4×10^7 bp; consequently, the per base mutation rates also vary over four orders of magnitude from 7×10^{-7} to 7×10^{-11}.

None of the microbes summarized in Drake et al. face intense, constant selective pressure on antigens imposed by vertebrate immunity – for example, it is unlikely that *Escherichia coli* depends on antigenic variation to avoid clearance from its hosts. It would be interesting to know if pathogens under very intense selection by host immunity have higher baseline mutation rates than related microbes under less intense immune pressure. High genome-wide mutation rates arise in bacteria by spontaneous mutator mutations, in which the mutator alleles raise the error rate during replication [38]. The mutator alleles probably are various DNA replication and repair enzymes. Ten or more genes of *E. coli* can develop mutator mutations. Assuming that each gene has about 1000 bases, the overall mutation rate of mutator loci is $10 \times 1000 \times 5 \times 10^{-10} \approx 10^{-6}$ to 10^{-5}, based on the per base mutation rate in given in Drake et al. [38]. Some mutations will be nearly neutral; others will cause extremely high mutation rates and will never increase in frequency. Typical *E. coli* cultures accumulate mutator mutants at a frequency of less than 10^{-5} [78], probably because most mutations are deleterious and therefore selection does not favor increased mutation rates. However, mutators can be strongly favored when the competitive conditions and the selective environment provide opportunities for the mutators to generate more beneficial mutations than the non-mutators [28,78]. In this case, mutators increase because they are linked with a higher frequency of beneficial mutations.

Although mutators are typically rare in freshly grown laboratory cultures, hospital isolates of *E. coli* and *Salmonella enterica* sometimes have mutator frequencies above 10^{-2} [51,65,73]. Extensive serial passage in the laboratory can also lead to high frequencies of mutators [124]. Thus, it appears that rapid change of hosts or culture conditions can increase the frequency of mutators 1000-fold relative to stable environmental conditions. As Drake et al. [38] point out, theory suggests that mutators can speed adaptation in asexual microbes [74,89,135]. It would be interesting to compare naturally occurring frequencies of mutators in stable and rapidly changing selective environments.

Targeting mutations to key loci would be more efficient than raising the genome-wide mutation rate. Various mechanisms can increase the mutation rate over short runs of nucleotides [47,111]. For example, *Streptococcus pyogenes* coats its surface with a variable M protein, of which 80 antigenically distinct variants are known [43,71]. The amino acid sequence of the M6 serotype revealed repeats in three regions of the protein [58,59].

Sequence analysis of variant M proteins suggests that mutations occur by generating both gains and losses of the duplications. These mutations probably arise by intragenic recombination between the DNA repeats, but may be created by slippage during replication. Slippage mutations over repeated DNA lead to gain or loss in the number of repeats and occur at frequencies much higher than typical replication errors [29]. The repeats of the M protein are multiples of three bases; thus changes in repeat number do not cause frameshift mutations. Some of the repeats vary slightly in base composition, so recombinations can alter sequence composition as well as total length. Fussenegger [47] reviews several other cases of bacterial cell-wall proteins that have repeated

sequences, most of which occur in multiples of 3 bp. Repeats are often associated with binding domains for other proteins or polysaccharides [145], so perhaps the ability to generate variable-length domains provides an advantage in attachment to host tissues or in escape from host immunity.

Apart from the well-known case of repeats and replication slippage, no evidence at present associates antigenic sites with higher replication errors. But this would certainly be an interesting problem to study further. One could, for example, focus on associations between mutation rate and nucleotide sequence. Comparison would be particularly interesting between epitopes that evolve rapidly and conserved regions of antigenic molecules that evolve slowly. Such comparison may help to identify aspects of nucleotide composition that promote higher error rates in replication.

In summary, microbial mutation rates per nucleotide decline with increasing genome size, causing a nearly constant mutation rate per genome per generation of about 0.003. Genome-wide hypermutation can raise the mutation rate at all sites within the genome. Such mutator phenotypes probably have altered replication enzymes. Low frequencies of mutator phenotypes have been observed in stable populations of *E. coli*, whereas fluctuating populations appear to maintain higher frequencies of mutators. In some cases, hypermutation may be targeted to certain genes by DNA repeats and other DNA sequence motifs that promote local replication errors.

15.3.2 Stochastic Switching Between Archival Copies

Many pathogens change critical surface molecules by switching expression between alternative genes. At least four types of switch mechanisms occur: replication errors that turn expression on or off, invertible promoters that change the direction of transcription, gene conversion into fixed expression sites, and transcriptional silencing of alternative genes.

15.3.2.1 Regulatory switches by replication errors of short repeats Short, repeated nucleotide sequences often lead to high error rates during replication. Repeats have recurring units typically with 1–5 bases per unit. Short, repeated DNA sequences probably lead to replication errors by slipped-strand mispairing [29,75,87]. Errors apparently arise when a DNA polymerase either skips forward a repeat unit, causing a deletion of one unit, or slips back one unit, producing a one-unit insertion.Gene expression can be turned on or off by insertions or deletions. Inserted or deleted repeats within the coding sequence cause frameshift mutations that prevent translation and production of a full protein. For example, the 11 opacity genes of *Neisseria meningitidis* influence binding to host cells and tissue tropism.These genes each have between eight and 28 CTCTT repeats, which can disrupt or restore the proper translational frame as the number of repeats changes [129,130].The limited repertoire of 11 genes and the crude on–off switching suggest that variable expression has more to do with altering cell tropism than with escape from host immunity [47].On–off switches can also be created by short repeats in transcriptional control regions. *Bordetella pertussis* controls expression of two distinct fimbriae by transcriptional switching [144]. Fimbriae are bacterial surface fibers that attach to host tissues. Particular cells produce both, only one, or neither of the fimbrial types. Sequences of about 15 C nucleotides in the transcriptional promoters of each of the two genes influence expression.The actual length of the poly-C sequence varies, probably by slipped-strand mispairing during replication. The length affects transcription of the attached gene. Thus, by the stochastic process of replication errors, the individual loci are turned on and off. Again, this sort of switching may have more to do with tissue tropism than with escape from immune recognition.

15.3.2.2 Invertible sequences *E. coli* stores two alternative fimbriae genes adjacent to each other on its chromosome [1]. A promoter region between the two genes controls transcription. The promoter triggers transcription in only one direction, thus expressing only one of the two variants. Occasionally, the promoter flips orientation, activating the alternative gene. The ends of the promoter have inverted repeats, which play a role in the recombination event that mediates the sequence inversion. *Salmonella* uses a similar mechanism to control flagellum expression [120].

Moraxella species use a different method to vary pilin expression [79,115]. The variable part of the pilin gene has alternate cassettes stored in adjacent locations. Inverted repeats flank the pair of alternate cassettes, causing the whole complex occasionally to flip orientation.The gene starts with an initial constant region and continues into one of the cassettes within the invertible complex.When the complex flips, the alternate variable cassette completes the gene. Several bacteriophage use a similar inversion system to switch genes encoding their tail fibers, which determine host range [61,66].

15.3.2.3 Gene conversion Some pathogens store many variant genes for a surface antigen, but express only one of the copies at any time. For example, there may be a single active expression site at which transcription occurs. Occasionally, one of the variant loci copies itself to the expression site by gene conversion – a type of intragenomic recombination that converts the target without altering the donor sequence. The genome preserves the archival library without change, but alters the expressed allele.

The spirochete *Borrelia hermsii* has approximately 30 alternative loci that encode an abundant surface lipoprotein [12]. There is a single active expression site when the spirochete is in mammalian hosts [13]. The expression site is changed by gene conversion to one of the variant archival copies at a rate of about 10^{-4} to 10^{-3} per cell division [14,131]. A small number of antigenic variants dominate the initial parasitemia of this blood-borne pathogen.The host then clears these initial variants with antibodies. Some of the bacteria from this

first parasitemia will have changed antigenic type. Those switches provide new variants that cause a second parasitemia, which is eventually recognized by the host and cleared. The cycle repeats several times, causing relapsing fever.

The protozoan *T. brucei* has hundreds of alternative loci that encode the dominant surface glycoprotein [16,105]. Typically, each cell expresses only one of the alternative loci. Switches in expression occur at a rate of up to 10^{-2} per cell division [138]. The switch mechanism is similar to that in *Borrelia hermsi* – gene conversion of archival copies into a transcriptionally active expression site. *T. brucei* has approximately 20 alternative transcription sites, of which only one is usually active. Thus, this parasite can also change expression by switching between transcription sites. It is not fully understood how different transcription sites are regulated.

15.3.2.4 Transcriptional silencing Changing transcriptional activation between different sites appears to be the mechanism by which *P. falciparum* regulates expression of its major surface antigen. *P. falciparum* expresses the *var* gene within erythrocytes. The gene product, PfEMP1, moves to the surface of the host cell, where it influences cellular adhesion and recognition by host immunity [37]. The *var* genes are highly diverse antigenically [133]. Each parasite exports only one PfEMP1 type to the erythrocyte surface, but a clone of parasites switches between PfEMP1 types [121]. Switching leads to a diverse population of PfEMP1 variants within a host and even wider diversity among hosts.It appears that many *var* loci are transcribed during the first few hours after erythrocyte infection, but only a single *var* gene transcript is active when PfEMP1 is translated and moved to the erythrocyte surface [30,118]. It may be that some mechanism shuts down expression of all but one locus without modifying the DNA sequence. Expression may be influenced by an interaction between an intron and the promoter, but the details need to be worked out [25,35].

15.3.3 Intragenomic Recombination

New variants of alternative genes in archival libraries may be created by recombination. For example, Rich et al. [110] found evidence for recombination between the archived loci of the variable short protein (Vsp) of *B. hermsii*. They studied the DNA sequences of 11 *vsp* loci within a single clone. These *vsp* loci are silent, archival copies that can, by gene conversion, be copied into the single expression site. The genes differ by 30–40% in amino acid sequence, providing sufficient diversity to reduce or eliminate antigenic cross-reactivity within the host.Rich et al. [110] used statistical analyses of *vsp* sequences to infer that past recombination events have occurred between archival loci. Those analyses focus on attributes such as runs of similar nucleotides between loci that occur more often than would be likely if alleles diverged only by accumulating mutations within each locus. Shared runs can be introduced into diverged loci by recombination.The archival antigenic repertoire of *T. brucei* evolves rapidly [105]. This species has a large archival library and multiple expression sites, but only one expression site is active at any time. New genes can be created within an active expression site when several donor sequences convert the site in a mosaic pattern [10,103]. When an active expression site becomes inactivated, the gene within that site probably becomes protected from further gene conversion events [102,104]. Thus, newly created genes by mosaic conversion become stored in the repertoire. Perhaps new genes in silent expression sites can move into more permanent archival locations by recombination, but this has not yet been observed. Recombination between silent, archived copies may also occur, which, although each event may be relatively rare, could strongly affect the evolutionary rate of the archived repertoire.

15.3.4 Mixing Between Genomes

New antigenic variants can be produced by mixing genes between distinct lineages. This happens in three ways.

Segregation brings together chromosomes from different lineages. Reassortment of influenza A's neuraminidase and hemagglutinin surface antigens provides the most famous example [70]. The genes for these antigens occur on two separate RNA segments of the genome – the genome has a total of eight segments. When two or more viruses infect a single cell, the parental segments all replicate separately and then are packaged together into new viral particles. This process can package the segments from different parents into a new virus. New neuraminidase–hemagglutinin combinations present novel antigenic properties to the host. Rare segregation events have introduced hemagglutinin from bird influenza into the genome of human influenza [143]. The novel hemagglutinins cross-reacted very little with those circulating in humans, allowing the new combinations to sweep through human populations and cause pandemics.

Intergenomic recombination occurs when chromosomes from different lineages exchange pieces of their nucleotide sequence. In protozoan parasites such as *Plasmodium* and certain *Trypanosoma* species (e.g., *brucei*), recombination happens as part of a typical Mendelian cycle of outcrossing sex [33,64]. Recombination can occur in viruses when two or more particles infect a single cell. DNA viruses may recombine relatively frequently because they can use the host's recombination enzymes [132]. RNA viruses may recombine less often because the host lacks specific enzymes to mediate reciprocal exchange of RNA segments. However, many descriptions of RNA virus recombination have been reported [72,112]. In all cases, even rare recombination can provide an important source for new antigenic variants. *Horizontal transfer* of DNA between bacteria introduces new nucleotide sequences into a lineage [97]. Transformation occurs when a cell takes up naked DNA from the environment. Some species transform at a particularly high rate, suggesting that they have specific adaptations for uptake and incorporation of foreign DNA [48]. For example, *Neisseria* species transform frequently enough to have many apparently mosaic genes

from interspecies transfers [48,127,148], and *N. gonorrhoeae* has low linkage disequilibrium across its genome [86]. Horizontal transfer also occurs when bacteriophage viruses carry DNA from one host cell to another or when two cells conjugate to transfer DNA from a donor to a recipient [97].

15.4 INTERACTIONS WITH HOST IMMUNITY

Specific immunity favors parasites that change their antigens and escape recognition. In this section, I summarize examples of parasite escape and the consequences for antigenic diversity within hosts.

15.4.1 Natural Selection of Antigenic Variants

In several pathogens, a changing profile of antigenic variants characterizes the course of infection within a single host. Natural selection favors variants that escape immune recognition, although escape is often temporary. Selection may also favor diversification of the pathogens for the ability to attack different types of host cells. I briefly summarize a few examples.

15.4.1.1 Simian immunodeficiency virus (SIV) and HIV Soudeyns et al. [126] identified the regions of the HIV-1 envelope under strong selective pressure by analyzing the pattern of nucleotide changes in the population. They compared the rate of non-synonymous d_n nucleotide replacements that cause an amino acid change versus the rate of synonymous d_s nucleotide replacements that do not cause an amino acid change. A high d_n/d_s ratio suggests positive natural selection favoring amino acid change; a low d_n/d_s ratio suggests negative natural selection opposing change in amino acids [100]. Soudeyns et al. [126] found that regions of the envelope gene under strong positive selection corresponded to epitopes recognized by CTLs. The non-synonymous substitutions in these epitopes typically abolished recognition by a matching CTL clone. The population of viruses accumulated diversity in the dominant epitopes over the course of infection within hosts. These results suggest that CTL attack based on specific recognition drives the rapid rate of amino acid replacements in these epitopes.

Kimata et al. [67] studied properties of SIV isolated from early and late stages of infection within individual hosts. The early viruses infected macrophages, replicated slowly, and the viral particles were susceptible to antibody-mediated clearance. The late viruses infected T cells, replicated more than 1000 times faster than early viruses, and were less sensitive to antibody-mediated clearance. Kimata et al. [67] did not determine the viral amino acid changes that altered cell tropism of SIV. Connor et al. [32] found that changes in the host-cell coreceptors used by early and late HIV-1 correlated with changes in cell tropism, but it is not yet clear which changes are essential for the virus's tropic specificity. Connor et al. [32] did show that the population of early viruses used a narrow range of coreceptors, whereas the late viruses were highly polymorphic for a diverse range of host coreceptors. Clearly, the virus is evolving to use various cell types.

The relative insensitivity of late SIV to antibody apparently depended on increased glycosylation of the envelope proteins. The late viruses with increased glycosylation were not recognized by antibodies that neutralized the early viruses. Viruses that escape antibody recognition gain significant advantage during the course of infection [27,116]. Kimata et al. [67] showed that, when injected into a naive host, the late SIV did not stimulate as much neutralizing antibody as did the early SIV. Additional glycosylation apparently reduces the ability of antibodies to form against the viral surface. Presumably, the glycosylation also hinders the ability of the virus to initiate infection; otherwise both early and late viruses would have enhanced glycosylation. Both the early, macrophage-tropic SIV and the late, T cell-tropic SIV used the host coreceptor CCR5 [67]. That observation contrasts with a study of early and late HIV-1 isolated from individual hosts, in which Connor et al. [32] found that early, macrophage-tropic viruses depended primarily on the CCR5 coreceptors, whereas the population of late viruses had expanded coreceptor use to include CCR5, CCR3, CCR2b, and CXCR4.

Many other studies focus on HIV diversification within hosts (e.g. [5,49,117]).

15.4.1.2 Hepatitis C virus (HCV) Farci et al. [41] obtained HCV samples at various stages of infection within individual hosts. They sequenced the envelope genes from these samples to determine the pattern of evolution within hosts. They then compared the evolutionary pattern with the clinical outcome of infection, which follows one of three courses: clearance in about 15% of cases; chronic infection and either slowly or rapidly progressive disease in about 85% of cases; and severe, fulminant hepatitis in rare cases.

Farci et al. [41] sampled three major periods of infection: the incubation period soon after infection; during the buildup of viremia but before significant expression of specific antibodies; and after the host's buildup of specific antibodies. The sequence diversity within hosts identified two distinct regions of the envelope genes. The hypervariable region evolved quickly and appeared to be under positive selection from the host immune system, whereas other regions of the envelope genes had relatively little genetic variation and did not evolve rapidly under any circumstances. Thus, the following comparisons focus only on the hypervariable region.

Those hosts that eventually cleared the virus had similar or higher rates of viral diversification before antibodies appeared than did those patients that developed chronic infection. By contrast, after antibodies appeared, chronic infection was correlated with significantly higher viral diversity and rates of evolution than occurred when the infection was eventually cleared. It appears that hosts who cleared the infection could contain viral diversity and eventually eliminate all variants, whereas those that progressed to chronic infection could not

control viral diversification. The rare and highly virulent fulminant pattern had low viral diversity and rates of evolution. This lack of diversity suggests either that the fulminant form may be associated with a single viral lineage that has a strong virulence determinant or that some hosts failed to mount an effective immune response.

15.4.1.3 Generality of within-host evolution of antigens

HIV and HCV share several characters that make them particularly likely to evolve within hosts. They are RNA viruses, which have relatively high mutation rates, relatively simple genomes, simple life cycles, potentially high replication rates, and potentially high population sizes within hosts. HIV and HCV also typically develop persistent infections with long residence times in each host. If the mutation rate per nucleotide per replication is 10^{-5} and the population of viruses is of the order of 10^{10} within a host, then there are 10^5 point mutations at every site in every generation. For every pair of sites, there will usually be at least one virus that carries mutations at both sites. Thus, there is a tremendous influx of mutational variation. Other RNA viruses such as influenza also have high mutation rates and potentially large populations within hosts, but the hosts typically clear infections within 2 weeks. Some within-host evolution very likely occurs, but it does not play a significant role in the infection dynamics within hosts. DNA-based pathogens produce much less mutational variation per replication. But large population sizes, long infection times, and hypermutation of epitopes could still lead to significant evolution within hosts. At present, the persistent RNA infections have been studied most intensively because of their obvious potential for rapid evolutionary change. As more data accumulate, it will be interesting to compare the extent and the rate of within-host evolutionary change in various pathogens.

15.4.2 Pathogen Manipulation of Host Immune Dynamics

Pathogens use several strategies to interfere with host immunity. A parasite's exposed surface antigens or candidate CTL epitopes may lack variation because the parasite can repel immune attack. I do not know of any evidence to support this idea, but it should be considered when studying candidate epitopes and their observed level of antigenic variation.

Several reviews summarize viral methods for reducing host immunity (e.g. [4,128]). Some bacteria also interfere with immune regulation [114]. I list just a few viral examples, taken from the outline given by Tortorella et al. [137].

Some viruses interfere with MHC presentation of antigens. Cases occur in which viruses reduce MHC function at the level of transcription, protein synthesis, degradation, transport to the cell surface, and maintenance at the cell surface. The host's natural killer (NK) cells attack other host cells that fail to present MHC class I molecules on their surface. Viruses that interfere with normal class I expression use various methods to prevent NK attack, for example, viral expression of an MHC class I homolog that interferes with NK activation.

Host cells often use programed suicide (apoptosis) to control infection. Various viruses interfere with different steps in the apoptosis control pathway.

The host uses cytokines to regulate many immune functions. Some viruses alter expression of host cytokines or express their own copies of cytokines. Other viruses express receptors for cytokines or for the constant (Fc) portion of antibodies. These viral receptors reduce concentrations of freely circulating host molecules or transmit signals that alter the regulation of host defense.

15.4.3 Sequence of Variants in Active Switching from Archives

Some parasites store alternative genes for antigenic surface molecules. Each individual parasite usually expresses only one of the alternatives [36,47]. Parasite lineages change expression from one stored gene to another at a low rate. In *T. brucei*, the switch rate is about 10^{-3} or 10^{-2} per cell division [138]. Antigenic switches affect the dynamics of the parasite population within the host. For example, the blood-borne bacterial spirochete *B. hermsii* causes a sequence of relapsing fevers [11,12]. Each relapse and recovery follows from a spike in bacterial density. The bacteria rise in abundance when new antigenic variants escape immune recognition and fall in abundance when the host generates a specific antibody response to clear the dominant variants. Switches between types within a cellular lineage occur stochastically. But the sequence of variants that dominate sequential waves of parasitemia tends to follow a repeatable order in *T. brucei* [15,50] and probably in *Borrelia* [14]. Temporal separation in the rise of different antigenic variants allows trypanosomes to continue an infection for a longer period of time [141]. If all variants rose in abundance early in the infection, they would all stimulate specific immune responses and be cleared, ending the infection. If the rise in different variants can be spread over time, then the infection can be prolonged. The puzzle is how stochastic changes in the surface antigens of individual parasites can lead to an ordered temporal pattern at the level of the population of parasites within the host [3,17,44,139,140]. Five hypotheses have been developed, none of which has strong empirical support at present. I briefly describe each idea.

First, the antigenic variants may differ in growth rate. Those that divide more quickly could dominate the early phases of infection, and those that divide more slowly could increase and be cleared later in the infection [119]. Computer studies and mathematical models show that variable growth rates alone cannot easily explain wide separation in the times of appearance of different variants [3,69]. Only with a very large spread in growth rates would the slowest variant be able to avoid an immune response long enough to develop an extended duration of total infection. Aslam and Turner [8] measured the growth rates of different variants of *T. brucei* and found little difference between the variants.

Second, parasite cells may temporarily express both the old and new antigens in the transition period after a molecular switch in antigenic type [3]. The double expressers could experience varying immune pressure depending on the time for complete antigenic replacement or aspects of cross-reactivity. This would favor some transitions to occur more easily than others, leading to temporal separation in the order of appearance for different antigenic variants. This model is rather complex and has gained little empirical or popular support, as discussed in several papers [2,18,19,21,90].

Third, the switch probabilities between antigenic variants may be structured in a way to provide sequential dominance and extended infection [44]. If the transition probabilities from each variant to the other variants are chosen randomly, then an extended sequence of expression does not develop because the transition pathways are too highly connected. The first antigenic types would generate several variants that develop a second parasitemia. Those second-order variants would generate nearly all other variants in a random switch matrix. The variants may arise in an extended sequence if the parasite structures the transition probabilities into separate sets of variants, with only rare transitions between sets. The first set of variants switches to a limited second set of variants, the second set connects to a limited third set, and so on. Longer infections enhance the probability of transmission to other hosts. Thus, natural selection favors the parasites to structure their switch probabilities in a hierarchical way in order to extend the length of infection. Paget-McNicol et al. [101] also developed a model in which switch rates vary, but did not consider how natural selection might modulate switch rates.

Fourth, Recker et al. [108] noted that hosts with stronger cross-reactive immune responses against *P. falciparum* variants are more likely to sustain chronic infections. Presumably, chronic infections mean that the parasite's repertoire of antigenic surface molecules can be structured into a pattern of sequential dominance. Based on these points, Recker et al. developed a model in which host immunity develops against two distinct components of the variable surface antigens. One part of the immune response develops lasting immunity against a unique component of each antigen. Another part of the host response develops short-lived immunity against a component of the antigenic molecule that is shared by other antigenic types. With these points in mind, imagine how a malarial infection would play out. One or a few antigenic variants dominate the initial parasitemia. The host develops specific immunity against each variant. One part of the immune response is specific for each variant and long-lived, clearing each variant and preventing another dominant wave of parasitemia by that variant. Another part of the immune response against a particular variant cross-reacts with many other variants – this cross-reactive component lasts only for a short while. As the initial parasitemia develops, some cells will have switched expression to other antigenic surface variants. As the first parasitemia clears, the next wave of parasitemia will develop from those rare variants that are least affected by the cross-reactive part of the host immune response. As those favored types develop into strong parasitemia, the process repeats, favoring in the subsequent wave those variants that cross-react least with the previous wave. Molineaux et al. [88] developed a more complex model of *P. falciparum* parasitemia dynamics and host immunity. Their model includes fitted values for how the various components of immunity clear parasites and variation in growth rate of different variants. This is an interesting analysis, but with so many parameters, it is difficult to determine whether the good fit with data arises from so many degrees of freedom or from a model that properly highlights the essential features of antigenic variation.

Turner [139] proposed a fifth explanation for high switch rates and ordered expression of variants. The parasite faces a trade-off between two requirements. On the one hand, competition between parasite genotypes favors high rates of switching and stochastic expression of multiple variants early in an infection. On the other hand, lower effective rates of switching later in an infection express variants sequentially and extend the total length of infection. Many *T. brucei* infections in the field probably begin with inoculation by multiple parasite genotypes transmitted by a single tsetse fly vector [77]. This creates competition between the multiple genotypes. According to Turner [139], competition intensifies the selective pressure on parasites to express many variants – variation allows escape from specific immunity by prior infections and helps to avoid cross-reactivity between variants expressed by different genotypes. These factors favor high rates of stochastic switching. The effective rate of switching drops as the infection progresses because the host develops immunity to many variants. Effective switches occur when they produce novel variants, and the rate at which novel variants arise declines over the course of infection. Those novel variants, when they do occur, can produce new waves of parasitemia, promoting parasite transmission.

Turner's idea brings out many interesting issues, particularly the role of competition between genotypes within a host. But his verbal model is not fully specified. For example, delayed expression of some variants and extended infection depend on the connectivity of transition pathways between variants, an issue he does not discuss. The problem calls for mathematical analysis coupled with empirical study. Connectivity of transition pathways between variants plays an important role in most theories. In Agur et al.'s [3] model, host immunity acting differentially on double expressers during the switch process favors some transitions over others. In Frank's [44] model, the different rates of molecular switching between variants provides structure to transition pathways. In Recker et al.'s [108] model, short-lived and cross-reactive host immunity favors particulars sequences of antigenic dominance. Turner's [139] model is not fully specified, but to work it must also provide a tendency for some transitions to be favored over others – this may occur by chance with random and rare switching or perhaps may favor common switches early and rare switches later in the sequence, more or less as in Frank's [44] model.

Connectivity of transition pathways has not been studied empirically. Frank and Barbour [46] have recently discussed this issue based on reanalysis of earlier data from *B. hermsii*.

15.5 EXPERIMENTAL EVOLUTION

Experimental evolution manipulates the environment of a population and observes the resulting pattern of evolutionary change. This allows one to study the selective forces that shape antigenic diversity. For example, one could manipulate immune selection by exposing parasites to different regimes of monoclonal antibodies. The parasites' evolutionary response reveals the adaptive potential and the constraints that shape patterns of antigenic variation.

In this chapter, I describe experimental evolution studies of foot-and-mouth disease virus (FMDV). I also use this virus as a case study to show how different methods combine to provide a deeper understanding of antigenic variation. These approaches include structural analysis of the virion, functional analysis of epitopes with regard to binding cellular receptors, sequence analysis of natural isolates, and experimental analysis of evolving populations.

15.5.1 Antigenicity and Structure of FMDV

FMDV is an RNA virus that frequently causes disease in domesticated cattle, swine, sheep, and goats [107]. FMDV populations maintain antigenic diversity in several rapidly evolving epitopes [42,85].

The most important epitopes occur on the GH loop of the VP1 surface protein [82,84,125]. This loop has about 20 amino acids that contribute to several overlapping epitopes. These antibody-binding sites appear to be determined mostly by the amino acids in the GH peptide (a continuous epitope). Antibodies that bind to an isolated GH peptide also neutralize intact viruses.

Many antibody escape variants occur in the GH loop, leading to extensive genetic variation in this region. However, a conserved amino acid triplet, Arg-Gly-Asp (RGD), also binds to antibodies. This conserved triplet mediates binding to integrin host-cell receptors typically used in FMDV attachment and entry [20,92,125]. The GH loop of VP1 contains continuous epitopes that together define the hypervariable antigenic site A common to all serotypes. Discontinuous epitopes occur when amino acid residues from widely separated sequence locations come together conformationally to form a binding surface for antibodies. Two antigenic sites of serotypes A, O, and C have discontinuous epitopes that have received widespread attention [42,84].

15.5.2 Antibody Escape Mutants

Many antibody escape mutants have been sequenced (references in [80]). One can develop a map of natural escape variants by comparing changes in sequence with differences in binding affinity to a panel of monoclonal antibodies (Mabs).

Two problems of interpreting selective pressures arise from an escape map based on natural variants. First, field isolates do not control the multitude of evolutionary pressures on variation. Mutants may spread either in direct response to antibody pressure, in response to other selective pressures, or by stochastic fluctuations independent of selective forces. Lack of variability may result either from lack of antibody pressure or from constraining selective pressures such as binding to host receptors.

The second problem for interpreting selective pressures from natural isolates concerns lack of control over genetic background. Whether a particular amino acid site affects antibody affinity may depend on conformation-changing variants at other sites.

Site-directed mutagenesis controls amino acid replacements in a fixed genetic background. One can alter sites that do not vary naturally to test for effects on antibody binding. Site-directed mutagenesis has provided useful information for FMDV [83]. But this method can only define changes in antibody binding; it does not show how viral populations actually respond to immune pressure. Several studies have applied monoclonal or polyclonal antibodies to FMDV in laboratory culture [82,125]. This allows direct control of selective pressure by comparing lines with and without exposure to antibodies. In addition, cultures can be started with genetically monomorphic viruses to control genetic background.

Martinez et al. [80] began laboratory evolution studies from a single viral clone of serotype C. These viruses were grown on baby hamster kidney cells (BHK-21). All host cells were derived from a single precursor cell. Two separate viral lines were established. C-S8c1 developed through three successive plaque isolations. C-S8c1p100 began with C-S8c1 and developed through 100 serial passages on a monolayer of BHK-21 cells. The host cells were refreshed from independent stock in each passage and therefore did not coevolve with the virus over the passage history.

In natural isolates, extensive sequence variability in the GH loop of VP1 correlates with escape from antibody neutralization. However, the Arg-Gly-Asp (RGD) sequence near the center of this GH loop is invariant in field isolates [125]. Controlled studies of laboratory evolution provide some insight into the evolution of this region.

The monoclonal antibody SD6 binds to an epitope spanning residues 136–147 in the GH loop of VP1. Martinez et al. [80] applied selective pressure by SD6 after establishment of the separate viral lines C-S8c1 and C-S8c1p100 by growing a cloned (genetically monomorphic) isolate in the presence of the antibody and sampling escape mutants. Nucleotide sequences of escape mutants were obtained. Each mutant (except one) escaped antibody neutralization by a single amino acid change. The different locations of these mutations in the original (C-S8c1) line compared with the serially passaged (C-S8c1p100) line provide the most striking result of this study. The original line conserved the Arg-Gly-Asp (RGD) motif at positions 141–143. By contrast, the serially passaged line had

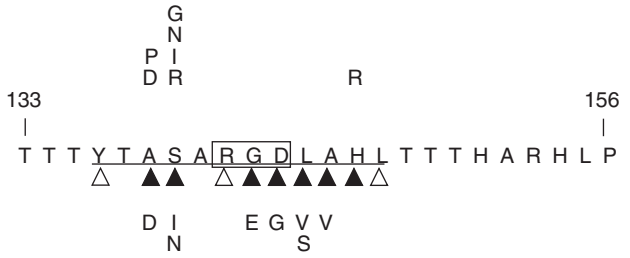

Fig. 15.1. Amino acid sequence in the central region of the VP1 GH loop of FMDV. The start and stop numbers label amino acid positions. The box shows the RGD motif at positions 141–143. The monoclonal antibody SD6 recognizes the continuous epitope defined by the underlined positions. Black triangles show positions at which most replacement amino acids greatly reduce binding by SD6; in other words, a single replacement at any of these sites creates an escape mutant. The white triangles denote positions that can tolerate certain amino acid replacements without greatly affecting antibody binding. Unmarked positions in the epitope can vary without much change in binding. The letters above the sequence summarize the escape mutants of C-S8c1 (original line); letters below the sequence summarize escape mutants of C-S8c1p100 (passaged line). Letters denote amino acids according to the standard single-letter code. Redrawn from [80].

numerous mutations within the RGD motif. Figure 15.1 contrasts the location of mutants for the two lines.

Variants in the RGD motif had not previously been observed in spite of neutralizing antibodies' affinity for this region. The RGD motif was thought to be invariant because of its essential role in binding to the host cell. Yet, the serially passaged line accumulated variants in this region. Those variants replicated with the same kinetics as the parental viruses of C-S8c1p100, with no loss in fitness. Baranowski et al. [9] showed that lineages with an altered RGD motif use an alternative pathway of attachment and entry to host cells.

Martinez et al. [80] sequenced the capsid genes from the original line, the serially passaged line, and an escape mutant of the serially passaged line. The escape mutant from the serially passaged line differed from the parental virus of this line only at a single site in the RGD region. Tolerance to replacements in the RGD region must follow from the differences accumulated by C-S8c1p100 during serial passage. Six amino acids differed between the original and serially passaged lines. Apparently, those substitutions changed cell tropism properties of the virus and allowed variation in the previously invariant RGD motif.

15.5.3 Cell Binding and Tropism

Attachment and entry to host cells impose strong natural selection on some regions of the viral surface. Experimental evolution provides one approach to analyze those selective forces, as described in the previous section. In this section, I briefly summarize further studies of amino acid variation in the FMDV capsid and the consequences for attachment and entry to host cells. Jackson et al. [62] compared the affinity of different viral genotypes for two integrin receptors, $\alpha_v\beta_3$ and $\alpha_5\beta_1$. The standard RGD motif was required for both receptors. The following amino acid at the RGD+1 position influenced relative affinity for the two integrin types. For $\alpha_v\beta_3$, several different amino acids at RGD+1 allowed binding, consistent with this receptor's multifunctional role in binding several ligands. By contrast, $\alpha_5\beta_1$ has narrower specificity, favoring a leucine at RGD+1. Jackson et al. [62] compared two viruses that differed only at RGD+1, the first with an arginine and the second with a leucine. The first virus had relatively higher affinity for $\alpha_v\beta_3$ compared with $\alpha_5\beta_1$. By contrast, the second virus had relatively higher affinity for $\alpha_5\beta_1$ compared with $\alpha_v\beta_3$. For at least some antibodies that recognize RGDL, loss of leucine at RGD+1 abolishes recognition (see Fig. 15.1) [80]. Thirty type O and eight type A field isolates had leucine at RGD+1. By contrast, five SAT-2 isolates had arginine, two Asia-1 isolates had methionine, and one Asia-1 isolate had leucine [62]. These and other data suggest that most serotypes have leucine at RGD+1 and perhaps a higher affinity for $\alpha_5\beta_1$. SAT-2 may either have greater affinity for $\alpha_v\beta_3$ or its binding may be conditioned by amino acid variants at other sites.

In another study, Jackson et al. [63] analyzed FMDV binding to a different integrin, $\alpha_v\beta_6$. This integrin binds relatively few host ligands and depends on an RGDLXXL motif with leucines at RGD+1 and RGD+4. Most FMDV isolates have leucines at those two positions. $\alpha_v\beta_3$ does not have stringent requirements at those sites, suggesting that $\alpha_v\beta_6$ may be an important natural receptor. Overall, RGDLXXL binds to the widest array of integrins, at least over those studied so far, although relative affinities for different integrins may be modulated by substitutions at RGD+1 and perhaps other sites. It would be interesting to sample isolates from various host tissues that differ in the densities of the various integrin receptors and analyze whether any substitutions appear relative to isolates in other body compartments of the same host.

Viral success in different cell types or in different hosts may depend on variations in nonstructural genes that do not mediate binding and entry to host cells. For example, Nunez et al. [95] serially passaged FMDV in guinea pigs. FMDV does not normally cause lesions in guinea pigs, but after serial passage, viral variants arose that caused disease. Among the several amino acid substitutions that arose during passage, a single change from glutamine to arginine at position 44 of gene 3A provided virulence. The function of 3A in FMDV is not known. In poliovirus, a distantly related picornavirus, 3A plays a role in virus-specific RNA synthesis. These studies show the potential power of experimental evolution in studying evolutionary forces, particularly when combined with analysis of naturally occurring variation.

15.5.4 Fitness Consequences of Substitutions

Antibody escape mutants are typically isolated in one of two ways. First, pathogens may be grown *in vitro* with antibodies. This creates selective pressure for substitutions that escape antibody recognition. Second, naturally occurring variants from field isolates may be tested against a panel of antibodies. Certain sets of antibodies may bind most isolates, allowing identification of those variants that differ at commonly recognized epitopes.

Escape variants gain a fitness advantage by avoiding antibody recognition targeted to important epitopes. However, those pathogen epitopes may also play a role in binding to host cells, in release from infected cells, or in some other aspect of the pathogen's life cycle. Functional and structural studies of amino acid substitutions provide one method of analysis. That approach has the advantage of directly assessing the mechanisms by which amino acid variants affect multiple components of parasite fitness, such as escape from antibody recognition and altered host attachment characteristics. Although functional and structural approaches can directly measure binding differences caused by amino acid substitutions in different genetic backgrounds, they cannot provide a good measure of all the fitness consequences associated with changes in genotype.

Carrillo et al. [26] used an alternative approach to analyze the consequences of amino acid substitutions. They studied the relative fitnesses *in vivo* of a parental FMDV genotype and three mutant genotypes derived from the parental type. They measured relative fitness by competing pairs of strains within live pigs. The parental type, C-S8c1, came from a C serotype isolated from a pig. The first monoclonal antibody-resistant mutant, MARM21, arose in a pig infected with C-S8c1. MARM21 differs from C-S8c1 by a single change from serine to arginine at VP1 139 (Fig. 15.1), providing escape from the monoclonal antibody SD6. The second mutant, S-3T_1, came from a blood sample of a pig 1 day after experimental inoculation with C-S8c1. That isolate had a single change from threonine to alanine at VP1 135 (Fig. 15.1). Only one of 58 monoclonal antibodies differentiated between the parental type and S-3T_1, and the difference in affinity was small. This supports the claim in Figure 15.1 that position 135 is not strongly antigenic. The third mutant, C-S15c1, derived from a field variant of type C1 isolated from a pig. This mutant type had eight amino acid differences in VP1 compared with C-S8c1. C-S15c1 did not react with monoclonal antibody SD6.

One of the three mutants was coinoculated with the parental type into each experimental pig. Two replicate pigs were used for each of the three pairs of mutant and parental types. Fever rose 1 day after infection and peaked 2 or 3 days post infection. All animals developed vesicular lesions 2–4 days post infection. For each animal, between two and seven samples were taken from lesions, and the relative proportions of the competing viruses were assayed by reactivity to monoclonal antibodies. The small sample sizes do not allow strong conclusions to be drawn. Rather, the following two results hint at what might be learned from more extensive studies of this sort. First, the parental type strongly dominated MARM21 in all seven lesions sampled from the two experimental animals, comprising between 80% and 94% of the viruses in each lesion. The MARM21 mutation appears to confer lower fitness *in vivo*, at least in the two animals tested. The lower fitness may arise because the mutant was cleared more effectively by antibodies, bound less efficiently to host cells, or had reduced performance in some other fitness component. Second, S-3T_1 abundance relative to the parental type varied strongly between lesions. In the two lesions analyzed from one animal, the parental type comprised 67 $\pm$ 3.4% and 3.2 $\pm$ 1.5% (mean $\pm$ standard deviation). In the other animal, the three lesions analyzed had parental-type percentages of 75 $\pm$ 4.1%%, 25 $\pm$ 2.8%, and 5.9 $\pm$ 1.2%. Differences in dominance between lesions also occurred between C-S15c1 and the parental type. Variations in dominance may arise from stochastic sampling of viruses that form lesions, from differences in tissue tropism, or from some other cause. Further studies of this sort may provide a more refined understanding of the multiple fitness consequences that follow from particular amino acid changes, their interactions with the genetic background of the virus, the role of different host genotypes, and the effect of prior exposure of hosts to different antigenic variants.

15.6 MEASURING SELECTION WITH POPULATION SAMPLES

Experimental evolution provides insight into kinetic and mechanistic aspects of parasite escape from host immunity. Such experimental studies clarify selective forces that influence change at certain amino acid sites. But experimental studies provide only a hint of what actually occurs in natural populations, in which selective pressures and evolutionary dynamics differ significantly from those in controlled laboratory studies. It is important to combine experimental insights with analyses of variation in natural populations. In this section, I discuss how population samples of nucleotide sequences provide information about natural selection of antigenic variation. I focus on themes directly related to the goal of this chapter – the synthesis between different kinds of biological analyses. In particular, I show how analysis of population samples complements studies of molecular structure and experimental evolution. Several books and articles review the methods to analyze population samples and the many different types of applications [23,34,56,60,68,76,91,93,94,98–100,113,147].

15.6.1 Positive and Negative Selection

The genetic code maps three sequential nucleotides (a codon) to a single amino acid or to a stop signal. The four different nucleotides combine to make $4^3 = 64$ different

codons. The 64 codons specify 20 different amino acids plus a stop signal, leading to an average of $64/21 ≈ 3 different codons for each amino acid or stop signal. This degenerate aspect of the code means that some nucleotide substitutions do not change the encoded amino acid or stop signal. Nucleotide substitutions that do not cause an amino acid change are called *synonymous*; those that do change the encoded amino acid are called *non-synonymous*. Synonymous substitutions do not affect the amino acid sequence and therefore should not be affected by natural selection of phenotype. By contrast, non-synonymous substitutions can be affected by selection because they do change the encoded protein. If there is no selection on proteins, then the same forces of mutation and random sampling influence all nucleotide changes, causing the rate of non-synonymous substitutions, d_n, to equal the rate of synonymous substitutions, d_s [76,93,100].

When natural selection favors change in amino acids, the non-synonymous substitution rate d_n rises. Thus, $d_n > d_s$ measured in a sample of sequences implies that natural selection has favored evolutionary change. This contribution of selection to the rate of amino acid change above the background measured by d_s is called *positive selection*. Parasite epitopes often show signs of positive selection as they change to escape recognition by host immunity [147].

By contrast, negative selection removes amino acid changes, preserving the amino acid sequence against the spread of mutations. Negative selection reduces the non-synonymous substitution rate, causing $d_n < d_s$. The great majority of sequences show negative selection, suggesting that most amino acid replacements are deleterious and are removed by natural selection. In cases where positive selection does occur, the non-synonymous replacements often cluster on protein surfaces involved in some sort of specific recognition. In these positively selected proteins, amino acid sites structurally hidden from external recognition often show the typical signs of negative selection.

15.6.2 Positive Selection to Avoid Host Recognition

Many examples of positive selection come from genes involved in host–parasite recognition [40,60,147]. These sequence analyses provide information about how selection has shaped the structure and function of proteins. For example, one may combine analysis of positive selection with structural data to determine which sites are exposed to antibody pressure. In the absence of structural data, sequences can be used to predict which sites are structurally exposed and can change and which sites are either not exposed or functionally constrained. I briefly summarize one example.

The tick-borne protozoan *Theileria annulata* causes disease in cattle [52]. The surface antigen Tams1 induces a strong antibody response and has been considered a candidate for developing a vaccine. However, Tams1 varies antigenically; thus studies have focused on the molecular nature of the variability to gain further insight. The structure and function of Tams1 have not been determined. Recently, Gubbels et al. [52] analyzed a population sample of nucleotide sequences to predict which domains of Tams1 change in response to host immunity and which domains do not vary because of structural or functional constraints. They found seven domains with elevated rates of non-synonymous substitutions compared with synonymous substitutions (Fig. 15.2), suggesting that these regions may be exposed to antibody pressure. Some domains had relatively little non-synonymous change, indicating that structural or functional constraints preserve amino acid sequence. These inferences provide guidance in vaccine design and point to testable hypotheses about antigenicity and structure.

15.6.3 Phylogenetic Analysis of Nucleotide Substitutions

Initial studies of selection often used small numbers of sequences, typically fewer than 100. Small sample sizes required aggregating observations across all nucleotide sites to gain sufficient statistical power. Conclusions focused on whether selection was positive, negative, or neutral when averaged over all sites. With slightly larger samples, one could do a sliding window analysis as in Figure 15.2 to infer the kind of selection averaged over sets of amino acids that occur contiguously in the two-dimensional sequence [40]. Major changes in binding and antigenicity often require only one or a few amino acid changes [45]. The analytical methods that

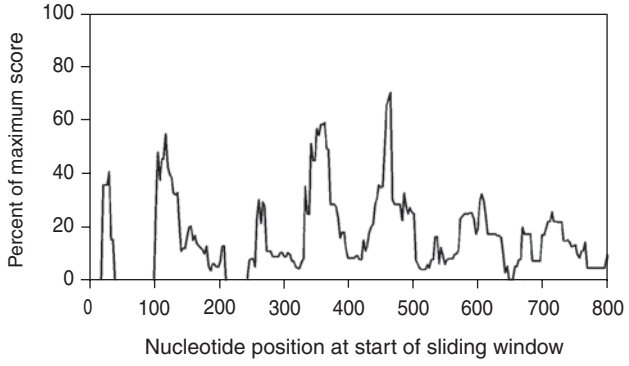

Fig. 15.2. The seven peaks identify the major regions of positive selection in the Tams1 protein. The 18 sequences analyzed in this figure have about 870 nucleotides. The analysis focused on a sliding window [40] of 60 nucleotides (20 amino acids). For each window shown on the *x*-axis, the numbers of non-synonymous and synonymous nucleotide substitutions were calculated by comparing the 18 sequences. The *y*-axis shows the strength of positive selection measured as follows. For each window of 60 nucleotides, each pair of sequences was compared. Each paired comparison was scored for the statistical significance of positive selection based on the numbers of non-synonymous and synonymous changes between the pair, with a score of zero for nonsignificant, a score of 1 for significant, and a score of 2 for highly significant. The maximum score is twice the number of comparisons; the actual score is the sum of significance values for each comparison; and the percentage of the maximum is the actual divided by the maximum multiplied by 100. From [52].

aggregate over whole sequences or sliding windows often fail to detect selection at the scale of single-site substitutions, which appears to be the proper scale for understanding antigenic evolution. Recently, larger samples of sequences have provided the opportunity to study the rates of synonymous and non-synonymous substitutions at individual nucleotide sites. Each individual substitution occurs within a lineal history of descent, that is, a change occurs between parent and offspring. To study each substitution directly, one must first arrange a sample of sequences into lineal relationships by building a phylogenetic tree. From the tree, one can infer the nucleotide sequence of ancestors, and therefore trace the history of each nucleotide change through time. Each nucleotide change can be classified as synonymous or non-synonymous. For each amino acid site, one can sum up the numbers of synonymous and non-synonymous nucleotide changes across the entire phylogeny and derive the associated rates of change. With appropriate statistics, one determines for each amino acid site whether non-synonymous changes occur significantly more or less often than synonymous changes [24,53,87,134,142,147]. The concepts of measuring positive and negative selection remain the same. However, for the first time, the statistical power has been raised to the point where analysis of population samples provides significant insight into the evolution of antigens. The power derives from studying the relative success of alternate amino acids at a single site. Important selective forces include the amino acids at other sites as well as binding properties to host immune molecules and other host receptors.

Haydon et al. [54] analyzed selection on individual amino acid sites of FMDV. Most sites showed mild to strong negative selection, as usually occurs. At 17 sites, they found evidence of significant positive selection. Twelve of these positively selected sites occurred at positions that had previously been observed to develop escape mutants in experimental evolution studies that imposed pressure by monoclonal antibodies. The other five sites indicate candidates for further experimental analysis.

Haydon et al.'s [54] study of natural isolates gives further evidence that a small number of amino acid sites determines a large fraction of antigenic evolution to escape antibody recognition. The combination of analyses on structure, experimental evolution, and natural variation provide an opportunity to study how complex evolutionary forces together determine the evolutionary dynamics of particular amino acids.

15.6.4 Predicting Evolution

Studies on positive selection in FMDV [54] and HIV [146] could not correlate amino acid substitutions at particular sites with the actual success of the viruses. In each case, selection was inferred strictly from the patterns of nucleotide substitutions in a sample of sequences.

Bush et al.'s [24] study of influenza takes the next step by associating particular amino acid substitutions with the success or failure of descendants that carry the substitutions. Influenza allows such studies because sequences have been collected each year over the past several decades, providing a history of which substitutions have led to success over time. The influenza data can be used to predict future evolution by two steps. First, previous patterns of substitutions and the successes of associated lineages suggest which amino acid sites contain variants that enhance fitness. Second, new variants arising at those key sites are predicted to be the progenitors of future lineages. Bush (this volume) discusses these methods applied to the influenza data.

15.7 SHAPE, CHARGE, BINDING KINETICS, AND EVOLUTION

The molecular shape and charge of host and parasite molecules influence binding of those molecules, which defines the nature of host–parasite recognition. Binding reactions determine the course of infection within each host, and the advantages and disadvantages of different antigenic variants of the parasite. Those advantages and disadvantages set the course of success for the different variants, changing the frequency of variants over time and space and determining the evolution of antigenic variation.

ABBREVIATIONS

AIDS:	Acquired immunodeficiency syndrome
CTL:	Cytotoxic T lymphocyte
FMDV:	Foot-and-mouth disease virus
HCV:	Hepatitis C virus
HIV:	Human immunodeficiency virus
Mabs:	Monoclonal antibodies
MHC:	Major histocompatibility complex
NK:	Natural killer
PfEMP1:	*Plasmodium falciparum* erythrocyte membrane protein
SIV:	Simian immunodeficiency virus

GLOSSARY

Antigen: A molecule that induces an immune response.

Antigenic variation: Molecular variation between individual parasites in a particular antigenic molecule, usually a specific host immune response directed against one variant does not recognize other variants as well.

Archival copies: Different genetic loci that store and do not express variant genes for an antigenic molecule.

Bacteremia: The presence or amount of bacteria in the blood.

Cross-reaction: The reaction of an antibody with an antigen other than the one that gave rise to it.

Cytoadherence: Strength of binding by a parasite to the surface of host cells.

Cytokine: Molecules secreted by certain cells of the immune system that have an effect on other cells.

Epitope, continuous, and discontinuous: The part of an antigen molecule to which an antibody attaches itself; continuous if the epitope is composed of a linear sequence of amino acids in the protein chain; discontinuous if, during protein folding, the epitope forms from disparate parts of the amino acid sequence.

Escape mutant: A genetic variant of a parasite epitope in which the original type was recognized by a particular host immune response and the mutant is not.

Glycosylation: The addition of molecular sugar components to a protein, sometimes protects an antigen from being recognized by the host immune response.

Macrophage: An immune cell that devours invading pathogens; stimulates other immune cells by presenting them with small pieces of the invader.

MHC class I: Molecules of the major histocompatibility complex that bind small peptides within cells and then present the MHC–peptide complex on the surface of cells for interaction with T cells; class I can stimulate cytotoxic T lymphocytes.

Monoclonal antibodies: An antibody produced by a single clone of cells and consisting of identical antibody molecules.

Parasitemia: The presence or amount of parasites in the blood.

Polyclonal antibodies: An antibody produced by a multiple distinct clones of cells and consisting of diverse, distinct antibody molecules.

Receptor: A particular molecule on a host cell surface to which a parasite binds.

Site-directed mutagenesis: Experimentally controlled mutation to a particular part of a gene, causing a mutational change in the targeted amino acid.

Tropism, cell: Tendency of a particular parasite variant to bind to a particular kind of cell.

Viremia: The presence or amount of viruses in the blood.

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CHAPTER 16

Hantavirus Coevolution with Their Rodent Hosts

Vincent Herbreteau,[1,2] Heikki Henttonen,[3] Kumiko Yoshimatsu,[4] Jean-Paul Gonzalez,[2] Yupin Suputtamongkol,[5] and Jean-Pierre Hugot[6]

[1]*Laboratoire Espace, Santé, Territoire; Université Paris X- Nanterre, 200 avenue de la république, 92000 Nanterre, France*

[2]*IRD, UT 178, Bangkok, Thailand*

[3]*Finnish Forest Research Institute, Vantaa Research Centre, POB18 FIN-01301 Vantaa, Finland*

[4]*Institute for Animal Experimentation, Hokkaido University, School of Medicine, Sapporo, Japan*

[5]*Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

[6]*Muséum National d'Histoire Naturelle, Département Systématique et Évolution, UMR Origine, Structure et Évolution de la Biodiversité, 75231 Paris Cedex 05, France*

"J'ai toujours pensé que le secret de la formation des espèces est dans leur morphologie, que les formes animales sont un langage hiéroglyphique dont on n'a pas la clef, et que l'explication du passé est tout entière dans des faits que nous avons sous les yeux, sans savoir les lire. Un jour viendra où la zoologie sera historique, c'est-à-dire où, au lieu de se borner à décrire la faune existante, elle cherchera à découvrir comment cette faune est arrivée à l'état où nous la voyons. Il se peut que les hypothèses de Darwin à ce sujet soient un jour jugées insuffisantes ou inexactes, mais sans contredit, elles sont dans la voie de la grande explication du monde et de la vraie philosophie."

Ernest Renan (1863).

"Since a long time, I believed that the secret explanation of the origin of species has to be found in their morphology. The animal forms are a hieroglyphic language remaining enigmatic and the whole explanation of the past stands in unreadable facts, which are before our very eyes. One day in the future, Zoology will become historical instead of being limited to the description of the existing fauna, and it will try to discover how this fauna happened to be as we can observe it now. Perhaps, Darwin's hypotheses on this subject may be considered inadequate or inaccurate in the future, however, they certainly are on the way of the main explanation of our world and of the true Philosophy."

Ernest Renan (1863).

16.1 INTRODUCTION

Genus hantavirus is one out of five genera within the Bunyaviridae family. The Bunyaviridae groups more than 350 species, most of which are arboviruses vectored by mosquitoes, ticks, sand flies, and so on. Within the family, only genus Tospovirus is a plant virus. Most of the other Bunyaviridae may cause human diseases. Bunyavirus is the agent of La Crosse encephalitis, and California encephalitis; Phlebovirus of Rift Valley fever (RVF), and sand fly fever; Nairovirus of Crimean-Congo hemorrhagic fever (CCHF); hantavirus of hemorrhagic fever with renal syndrome (HFRS), or hantavirus pulmonary syndrome (HPS).

Hantaviruses, which are usually hosted by wild mammals (rodents and shrews), are potentially pathogenic for humans. Several serologically distinct virus species, associated with

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different syndromes, have been recognized. In Asia, Hantaan, Dobrava, Seoul, and Puumala cause the clinical forms of HFRS [36]. In South America, Sin Nombre and Andes are responsible for HPS [5]. A last group, Tula, widely distributed in Russia and Eastern Europe has never been associated with a human disease. Muridae rodents are the primary reservoir and, because each virus group is associated with a particular rodent family, the hypothesis of coevolution with cophylogeny has been suggested [7,24,25,36].

Coevolution may be defined as: the mutual evolutionary influence between two species: each of the species exerts selective pressure on the other, so they evolve together. Coevolution is an extreme example of mutualism and may be described as a change in the genetic composition of one species (or group) in response to a genetic change in another. Coevolution may be considered among broad groups of taxa, and because all interacting organisms bring about changes, initially it might seem that everything is involved in coevolution. However, some particular situations allow more accurate observation of the phenomenon: this is when coevolution is going on between pairs of species from each group. Patterns of paired coevolution are particularly frequent in the evolution of hosts and parasites. If the term is usually attributed to Ehrlich and Raven's study of butterflies on plants [8], the idea was very apparent in the "Origin of Species." Since Darwin, many authors have suggested that the phylogenetic relationships of highly host specific parasites would provide valuable information about the evolutionary history of their hosts [3]. This is because, sometimes, the life histories of two different lineages are so intimately linked that a speciation in one group induces a parallel speciation event in the other. In such a case, comparison of host cladograms and parasites is crucial. If they are congruent, this certainly suggests coevolutionary phenomena and "association by descent." Nevertheless, cophylogeny does not mean reciprocal phenomena: speciation of the host may induce the speciation of the parasite without parasite-induced speciation of the host. One needs to know the evolutionary history before deciding which type of co-"evolution" is observed. This means that phylogenetic analysis is necessary and constitutes the first step in this type of study.

Viruses are the "achieved" parasites: they are completely dependent on the cell machinery of their host, hijacked for their own profit. Conversely, their action on the survival of the host may deeply influence its evolution. Thus, they present all the opportunities for reciprocal influences on evolution.

16.2 GENERALITIES ON HANTAVIRUSES

Hantaviruses are a relatively newly discovered genus of virus. First isolated between North and South Korea in 1976, they were named for the Hantaan River, which delineates the endemic area shared by the two countries. However, it is now thought that hantaviruses have been infecting rodent populations for thousands of years and possibly humans since the beginning of the twentieth century, in different regions of the world. hantaviruses (genus hantavirus, family Bunyaviridae) are a group of at least 25 antigenically distinct viruses carried in rodents. Some of these viruses can cause hemorrhagic fever with renal syndrome and HPS in humans. HFRS is a group of clinically similar diseases that occur throughout Eurasia. HFRS includes several diseases that formerly had other names, including Korean hemorrhagic fever, epidemic hemorrhagic fever, and nephropathia epidemica. hantaviruses that can cause HFRS include Hantaan virus, Puumala virus, Dobrava virus, and Seoul virus.

16.2.1 Hantavirus Taxonomy

In the Eight Report of the International Committee on Taxonomy of Viruses [26], the family Bunyaviridae is subdivided into five genera: Orthobunyavirus, hantavirus, Nairovirus, Phlebovirus, and Tospovirus. Within genus hantavirus, 22 different species are recognized. In each virus species different strains, from one to eight, may be identified and named. Also, abbreviations are given for certain strains. Different criterions are used to decide which strain may be considered a distinct species. One of them is whether the primary rodent reservoir is a particular species or subspecies. We agree with this, and we have used this criterion to choose the different virus strains included in this study. However, the list given in the Eighth Report [26] shows that all the strains corresponding to this criterion are not considered to be distinct virus species. For instance, Andes or Sin Nombre include several strains, each one having a different hosts species and sometimes a different geographic origin. Also, some of these strains are assigned a particular abbreviation and others are not. Thus, there is no exact correspondence between the given species statute, the attribution of a particular abbreviation, the specificity for a particular host, or a particular geographic range. This is probably because quantitative criterions are also applied and are considered predominant. For instance, the report assess that "Species exhibit at least 7% difference in amino acid identity on comparison of the complete glycoprotein precursor and nucleocapsid protein sequences." Thus, in the following paragraph and in the figures:

- Virus species listed in the Eighth Report [26] are in italic script.
- Strain names are in roman script, or are represented using their abbreviation in caps when an abbreviation has been proposed.
- When different strains of a same virus species are included, a number or an adjective (generally dealing with the geographic origin) is added.
- The correspondence between the virus species, strain names, and abbreviations is given in Table 16.1.
- The main clades are named using the dominant virus species name (in black italic script); when different virus species are included in a same clade, or when the cladistic analysis suggests that several strains or species may be grouped together, a species name is proposed (in black italic script).

Table 16.1 consists of a list of hantaviruses included in the present study. First two columns: name of virus species and/or

TABLE 16.1. List of Hantaviruses Included in Present Study.

	Virus Species and Strain	Abbreviation	Host Species	Family	Accession no.	Nucl	Region	Distribution	Reference
1	*Dobrava*/Estonia	DOBV-Estonia1	*Apodemus agrarius*	Murinae	AJ009773	1671	PAL	Estonia (Saaremaa)	J. Gen. Virol. 80 (Pt 2), 371-379 (1999)
2	*Dobrava*/Estonia	DOBV-Estonia2	*Apodemus agrarius*	Murinae	AJ009775	1671	PAL	Estonia (Saaremaa)	J. Gen. Virol. 80 (Pt 2), 371-379 (1999)
3	*Dobrava*/Slovakia	DOBV-Slovakia1	*Apodemus agrarius*	Murinae	AJ269549	1704	PAL	Slovakia (Kosice)	J. Med. Virol. 63 (2), 158-167 (2001)
4	*Dobrava*/Bosnia	DOBV-Bosnia	*Apodemus flavicollis*	Murinae	L41916	1670	PAL	Bosnia	J. Gen. Virol. 76 (Pt 11), 2801-2808 (1995)
5	*Dobrava*/Greece	DOBV-Greece1	*Apodemus flavicollis*	Murinae	AJ410615	1290	PAL	Greece (Northeast)	J. Med. Virol. 69 (3), 408-416 (2003)
6	*Dobrava*/Greece	DOBV-Greece2	*Apodemus flavicollis*	Murinae	AJ410619	1290	PAL	Greece (Northeast)	J. Med. Virol. 69 (3), 408-416 (2003)
7	*Dobrava*/Russia	DOBV-Russia1	*Apodemus flavicollis*	Murinae	AF442623	1637	PAL	Russia (Krasnodar)	Dekonenko, A. 2001
8	*Dobrava*/Russia	DOBV-Russia2	*Apodemus sp.*	Murinae	AF442622	1196	PAL	Russia (Goryachiy)	Dekonenko, A. 2002
9	*Dobrava*/Slovakia	DOBV-Slovakia2	*Apodemus sp.*	Murinae	AJ269550	1704	PAL	Slovakia (Kosice)	J. Med. Virol. 63 (2), 158-167 (2001)
10	*Hantaan*/76118	HTNV-76118	*Apodemus sp.*	Murinae	M14626	1696	PAL	South Korea	[39]
11	*Hantaan*/Maaji	HTNV-Maaji	*Apodemus agrarius*	Murinae	AF321094	1700	PAL	Korea (Maaji)	Virus Genes 21 (3), 227-232 (2000)
12	*Hantaan*/Amur AP61	AMRV.AP61	*Apodemus peninsulae*	Murinae	AB071183	1290	PAL	Russia FE (Solovey)	Emerging Infect. Dis. 8 (8), 768-776 (2002)
13	*Hantaan*/Amur AP63	AMRV.AP63	*Apodemus peninsulae*	Murinae	AB071184	1696	PAL	Russia FE (Solovey)	Emerging Infect. Dis. 8 (8), 768-776 (2002)
14	*Hantaan*/Guizhou	HTNV-Guizhou	*Apodemus sp.*	Murinae	AB027097	1635	PAL	China (Guizhou)	Virology 278 (2), 332-345 (2000)
15	*Hantaan*/Da Bie Shan	DBSV	*Niviventer confucianus*	Murinae	AB027523	1654	PAL	China (Anhui)	Virology 278 (2), 332-345 (2000)
16	***Hantaan*/Bat**	**HTNV-Bat**	***Rhinolophus ferrumequinum***	**Rinolophidae**	**U37768**	**1696**	**PAL**	**Korea**	**Kim, G.-R. and Jung,Y.-T. 1995**
17	*Seoul*/L99	SEOV-L99	*Rattus losea*	Murinae	AF288299	1764	PAL	China (Jiangxi)	Zhihui,Y. et al. (2000)
18	*Seoul*/Sapporo	SEOV-Sapporo	*Rattus norvegicus*	Murinae	M34881	1769	PAL	Japan (Sapporo)	Virology 176 (1), 114-125 (1990)
19	*Seoul*/Shanxi	SEOV-Shanxi	*Rattus rattus*	Murinae	AF288643	1772	PAL	China (Shanxi)	Yao, Z. et al., 2000
20	*Seoul*/Tchoupitoulas	SEOV-Tchoupi	*Rattus rattus*	Murinae	AF329389	1785	NEA	USA (Louisiana)	Yao, Z. et al. 2000
21	*Seoul*/Zhejiang	SEOV-lZhejiang1	*Rattus rattus*	Murinae	AB027522	1692	PAL	China (Zhejiang)	Virology 278 (2), 332-345 (2000)
22	*Seoul*/Zhejiang	SEOV-Zhejiang2	*Rattus rattus*	Murinae	AF288653	1772	PAL	China (Zhejiang)	Yao, Z. et al. 2000

(Continued)

TABLE 16.1. (*Continued*)

	Virus Species and Strain	Abbreviation	Host Species	Family	Accession no.	Nucl	Region	Distribution	Reference
23	*Sin Nombre*	SNV	*Peromyscus maniculatus*	Neotominae	L25784	2059	NEA	USA (S-West & Central)	Virology 200 (2), 715-723 (1994)
24	*Sin Nombre*/Convict Creek	SNV-Conv.74	*Peromyscus maniculatus*	Neotominae	L33683	1287	NEA	USA (California)	Virology 206 (2), 963-972 (1995)
25	*Sin Nombre*/Convict Creek	SNV-Conv.107	*Peromyscus maniculatus*	Neotominae	L33816	1287	NEA	USA (California)	Virology 206 (2), 963-972 (1995)
26	*Sin Nombre*/ Monongahela	MGLV	*Peromyscus maniculatus*	Neotominae	U32591	2082	NEA	USA (Appalachian)	J. Gen. Virol. 76 (Pt 12), 3195-3199 (1995)
27	*New York*/RI1	NYV-RI1	*Peromyscus leucopus*	Neotominae	U09488	2078	NEA	USA (North East)	J. Med. Virol. 46 (1), 21-27 (1995)
28	Limestone Canyon	LimCanyon	*Peromyscus boylii*	Neotominae	AF307322	1209	NEA	USA (Arizona)	Virology 286 (2), 345-353 (2001)
29	*El Moro Canyon*	ELMCV	*Reithrodontomys megalotis*	Neotominae	U11427	1896	NEA	USA (New Mexico)	[15]
30	Rio Segundo	RioSegundo	*Reithrodontomys mexicanus*	Neotominae	U18100	1749	NEO	Costa Rica	Virology 207 (2), 452-459 (1995)
31	*Andes*/AH1	ANDV-AH1	*Oligoryzomys longicaudatus*	Sigmodontinae	AF004660	1876	NEO	Argentina	[21]
32	*Andes*/Bermejo	BMJV	*Oligoryzomys chacoensis*	Sigmodontinae	AF482713	1933	NEO	Argentina (Oran)	J. Virol. 76 (8), 3765-3773 (2002)
33	*Andes*/Chile	ANDV-Chile1	*Oligoryzomys longicaudatus*	Sigmodontinae	AF291702	1871	NEO	Chile (Aysen)	J. Virol. 76 (8), 3765-3773 (2002)
34	*Andes*/Chile	ANDV-Chile2	*Oligoryzomys longicaudatus*	Sigmodontinae	NC003466	1871	NEO	Chile (Aysen)	J. Virol. 76 (8), 3765-3773 (2002)
35	*Andes*/Lechiguanas	LECV	*Oligoryzomys flavescens*	Sigmodontinae	AF482714	1938	NEO	Argentina (Lechiguana)	[20]
36	*Andes*/Norte	ANDV-Norte	*Oligoryzomys chacoensis*	Sigmodontinae	AF325966	1921	NEO	Argentina Norte	Am. J. Trop. Med. Hyg. 66 (6), 713-720 (2002)
37	*Andes*/Oran	ORNV	*Oligoryzomys longicaudatus*	Sigmodontinae	AF482715	1919	NEO	Argentina (Oran)	J. Virol. 76 (8), 3765-3773 (2002)
38	*Andes*/Pergamino	PRGV	*Akodon azarae*	Sigmodontinae	AF482717	1860	NEO	Argentina	J. Virol. 76 (8), 3765-3773 (2002)
39	Maciel	Maciel	*Bolomys benefactus*	Sigmodontinae	AF482716	1869	NEO	Argentina (Maciel)	J. Virol. 76 (8), 3765-3773 (2002)
40	*Laguna Negra*	LANV	*Calomys laucha*	Sigmodontinae	AF005727	1904	NEO	Paraguay, Bolivia	Virology 238 (1), 115-127 (1997)
41	Rio Mamore	RioMamore	*Oryzomys microtis*	Sigmodontinae	U52136	1975	NEO	Bolivia	Am. J. Trop. Med. Hyg. 57 (3), 368-374 (1997)
42	*Bayou*	BAYV	*Oryzomys palustris*	Sigmodontinae	L36929	1958	NEA	USA (Louisiana)	J. Virol. 69 (3), 1980-1983 (1995)
43	*Black Creek Canal*	BCCV	*Sigmodon hispidus*	Sigmodontinae	L39949	1989	NEA	USA (Florida)	J. Virol. 69 (3), 1980-1983 (1995)

44	*Muleshoe*	MULV	*Sigmodon hispidus*	Sigmodontinae	U54575	1989	NEA	USA (Texas)	Am. J. Trop. Med. Hyg. 55 (6), 672-679 (1996)
45	*Caño Delgadito*	CADV	*Sigmodon alstoni*	Sigmodontinae	AF000140	1130	NEO	Venezuela (Portuguesa)	Fulhorst,C.F., et al. 1997
46	*Isla Vista*	ISLAV-1	*Microtus californicus*	Arvicolinae	U19302	1720	NEA	USA (California)	J. Gen. Virol. 76, 3195-3199 (1995)
47	*Isla Vista*	ISLAV-2	*Microtus californicus*	Arvicolinae	U31534	1720	NEA	USA (California)	J. Gen. Virol. 76, 3195-3199 (1995)
48	*Isla Vista*	ISLAV-3	*Microtus californicus*	Arvicolinae	U31535	1302	NEA	USA (California)	J. Gen. Virol. 76, 3195-3199 (1995)
49	*Prospect Hill*	PHV-1	*Microtus montanus*	Arvicolinae	M34011	1675	NEA	USA	Virology 175 (1), 167-175 (1990)
50	*Prospect Hill*	PHV-2	*Microtus montanus*	Arvicolinae	Z49098	1675	NEA	USA	[30]
51	Prairie Vole	PrairieVole	*Microtus ochrogaster*	Arvicolinae	U19303	1722	NEA	USA (?)	Song,W., et al. 1995
52	*Topografov*	TOPV	*Lemmus sibiricus*	Arvicolinae	AJ011646	1951	PAL	Russia FE (Taymyr)	J. Virol. 73 (7), 5586-5592 (1999)
53	*Khabarovsk*	KHAV	*Microtus fortis*	Arvicolinae	U35255	1845	PAL	Russia FE (Khabarovsk)	[16]
54	Vladivostock	Vladivostock	*Microtus fortis*	Arvicolinae	AB011630	1228	PAL	Russia FE (Vladivostok)	Kariwa,H., et al. 1998
55	*Tula*/Germany1	TULV-Germany1	*Microtus arvalis*	Arvicolinae	AF164093	1832	PAL	Germany	Scharninghausen,J.J., et al. 1999
56	*Tula*/Germany2	TULV-Germany2	*Microtus arvalis*	Arvicolinae	AF289821	1828	PAL	Germany	Leitmeyer,K.C., et al. 2000
57	*Tula*/Lodz	TULV-Lodz1	*Microtus arvalis*	Arvicolinae	AF063892	1852	PAL	Poland	Song,J.-W., et al. 1995
58	*Tula*/Lodz	TULV Lodz2	*Microtus arvalis*	Arvicolinae	AF063897	1852	PAL	Poland	Song,J.-W., et al., 1995
59	*Tula*/Moravia	TULV-Moravia	*Microtus arvalis*	Arvicolinae	Z69991	1831	PAL	Moravia	J. Gen. Virol. 77 (Pt 12), 3063-3067 (1996)
60	*Tula*/Slovakia	TULV-Slvk1	*Microtus arvalis*	Arvicolinae	AJ223601	1831	PAL	Slovakia (Koziky)	J. Virol. 73 (1), 667-675 (1999)
61	*Tula*/Slovakia	TULV-Slvk2	*Microtus arvalis*	Arvicolinae	AJ223600	1831	PAL	Slovakia (Koziky)	J. Virol. 73 (1), 667-675 (1999)
62	*Tula*/Slovakia	TULV-Slvk3	*Microtus arvalis*	Arvicolinae	Z48235	1831	PAL	Slovakia (Malacky)	Virus Genes 10 (3), 277-281 (1995)
63	*Tula*/Slovakia	TULV-Slvk4	*Microtus arvalis*	Arvicolinae	Y13979	1833	PAL	Slovakia (Kosice)	J. Virol. 73 (1), 667-675 (1999)
64	*Tula*/Slovakia	TULV-Slvk5	*Microtus arvalis*	Arvicolinae	Y13980	1832	PAL	Slovakia (Kosice)	J. Virol. 73 (1), 667-675 (1999)
65	*Tula*/Slovakia	TULV-Slvk6	*Microtus arvalis*	Arvicolinae	Z68191	1831	PAL	Slovakia (Malacky)	Virus Genes 10 (3), 277-281 (1995)

(Continued)

TABLE 16.1. (*Continued*)

	Virus Species and Strain	Abbreviation	Host Species	Family	Accession no.	Nucl	Region	Distribution	Reference
66	*Tula*/Russia	TULV-Russia	*Microtus gregalis*	Arvicolinae	Z30941	1847	PAL	Russia (Tula)	J. Virol. 68 (12), 7833-7839 (1994)
67	*Tula*/Serbia	TULV-Serbia	*Microtus subterraneus*	Arvicolinae	AF017659	1834	PAL	Serbia (Cacac)	Song,J.-W., et al. 1997
68	*Puumala*/Bashkortostan	PUUV-Bashkor	*Clethrionomys glareolus*	Arvicolinae	AF442613	1733	PAL	Russia (Bashkortostan)	Dekonenko,A. and Khasanova,S. (2001)
69	*Puumala*/Belgium	PUUV-Belgium	*Clethrionomys glareolus*	Arvicolinae	AJ277030	1837	PAL	Belgium (Thuin)	Escutenaire S. (2001)
70	*Puumala*/CG1820	PUUV-CG1820	*Clethrionomys glareolus*	Arvicolinae	M32750	1784	PAL	?	Virology 174 (1), 79-86 (1990)
71	*Puumala*/Denmark	PUUV-Denmark	*Clethrionomys glareolus*	Arvicolinae	AJ238791	1831	PAL	Denmark	J. Gen. Virol. 81 (Pt 12), 2833-2841 (2000)
72	*Puumala*/Evo	PUUV-Evo	*Clethrionomys glareolus*	Arvicolinae	Z30703	1832	PAL	Finland	Virus Res. 38 (1), 25-41 (1995)
73	*Puumala*/Kamiiso	HOKV-Kamiiso	*Clethrionomys rufocanus*	Arvicolinae	AB010730	1833	PAL	Japan (Hokkaido)	Virus Res. 59 (2), 219-228 (1999)
74	*Puumala*/Japan	HOKV-Japan	*Clethrionomys rufocanus*	Arvicolinae	AB010731	1833	PAL	Japan (Tobetsu)	Virus Res. 59 (2), 219-228 (1999)
75	*Puumala*/Karelia	PUUV-Karelia1	*Clethrionomys glareolus*	Arvicolinae	AJ238790	1832	PAL	Russia (Karelia, Gomselga)	J. Gen. Virol. 81 (Pt 12), 2833-2841 (2000)
76	*Puumala*/Karelia	PUUV-Karelia2	*Clethrionomys glareolus*	Arvicolinae	AJ238788	1828	PAL	Russia (Karelia, Karhumaki)	J. Gen. Virol. 81 (Pt 12), 2833-2841 (2000)
77	*Puumala*/Karelia	PUUV-Karelia3	*Clethrionomys glareolus*	Arvicolinae	AJ238789	1830	PAL	Russia (Karelia, Kolodozero)	J. Gen. Virol. 81 (Pt 12), 2833-2841 (2000)
78	*Puumala*/Kazan	PUUV-Kazan	*Clethrionomys glareolus*	Arvicolinae	Z84204	1826	PAL	Sweden?	J. Virol. 71 (12), 9515-9523 (1997)
79	*Puumala*/Norway	PUUV-Norway1	*Clethrionomys glareolus*	Arvicolinae	AJ223369	1849	PAL	Norway (Eidsvoll)	J. Gen. Virol. 79 (Pt 11), 2603-2614 (1998)
80	*Puumala*/Norway	PUUV-Norway3	*Clethrionomys glareolus*	Arvicolinae	AJ223374	1828	PAL	Norway (Mellansel)	J. Gen. Virol. 79 (Pt 11), 2603-2614 (1998)
81	*Puumala*/Norway	PUUV-Norway4	*Clethrionomys glareolus*	Arvicolinae	AJ223375	1829	PAL	Norway (Mellansel)	J. Gen. Virol. 79 (Pt 11), 2603-2614 (1998)
82	*Puumala*/Norway	PUUV-Norway5	*Clethrionomys glareolus*	Arvicolinae	AJ223376	1871	PAL	Norway (Solleftea)	J. Gen. Virol. 79 (Pt 11), 2603-2614 (1998)
83	*Puumala*/Norway	PUUV-Norway6	*Clethrionomys glareolus*	Arvicolinae	AJ223377	1882	PAL	Norway (Solleftea)	J. Gen. Virol. 79 (Pt 11), 2603-2614 (1998)
84	*Puumala*/Norway	PUUV-Norway7	*Clethrionomys glareolus*	Arvicolinae	AJ223380	1827	PAL	Norway (Tavelsjo)	J. Gen. Virol. 79 (Pt 11), 2603-2614 (1998)
85	*Puumala*/Omsk	PUUV-Omsk1	*Clethrionomys glareolus*	Arvicolinae	AF367067	1732	PAL	Omsk-Russia (W Siberia)	Dekonenko,A., et al. 2001
86	*Puumala*/Omsk	PUUV-Omsk2	*Clethrionomys glareolus*	Arvicolinae	AF367068	1732	PAL	Omsk-Russia (W Siberia)	Dekonenko,A., et al. 2001

87	*Puumala*/Omsk	PUUV-Omsk3	*Clethrionomys glareolus*	Arvicolinae	AF367069	1732	PAL	Omsk-Russia (W Siberia)	Dekonenko,A., et al. 2001
88	*Puumala*/Omsk	PUUV-Omsk4	*Clethrionomys glareolus*	Arvicolinae	AF367070	1732	PAL	Omsk-Russia (W Siberia)	Dekonenko,A., et al. 2001
89	*Puumala*/Slovakia	PUUV-Slovakia	*Clethrionomys glareolus*	Arvicolinae	AF294652	1809	PAL	Slovakia	Leitmeyer,K.C., et al. 2000
90	*Puumala*/Sotkamo	PUUV-Sotkamo	*Clethrionomys glareolus*	Arvicolinae	X61035	1830	PAL	Finland (Sotkamo)	J. Gen. Virol. 73 (Pt 4), 829-838 (1992)
91	*Puumala*/Udmurtia	PUUV-Udmurtia	*Clethrionomys glareolus*	Arvicolinae	Z21497	1827	PAL	Finland (Udmurtia)	J. Gen. Virol. 75 (Pt 2), 405-409 (1994)
92	*Puumala*/Vranica	PUUV-Vranica	*Clethrionomys glareolus*	Arvicolinae	U14137	1828	PAL	Bosnia (Vranica)	Arch. Virol. 140 (11), 2011-2026 (1995)
93	***Thottapalayam***	**Thottalayam**	***Suncus murinus***	**Soricidae**	**AY526097**	**1530**	**ORIENT**	**India (Thottalayam)**	**Schmaljohn,C.S. and Toney,A. (2004)**

strain and abbreviation used in text and figures. Columns 3 and 4: scientific and Family names of principal host. Column 5 and 6: accession number of GenBank sequence, number of nucleotides given. Column 7: biogeographic areas, Palearctic (PAL), Nearctic (NEA), Neotropical (NEO), and Oriental (ORIENT). Column 8: country, and when possible province or locality, where virus strain has been collected. Column 9: reference of original publication; name of authors and year in case of direct submission.

16.2.2 Geographic Distribution

Hantaviruses have a large geographic distribution. Most of the recorded species were collected in the Holarctic and the Neotropics (Northern Asia, Europe, North America, and South America). Two species were isolated from South Asia [4]: Thottapalayam from a shrew (*Suncus murinus*) in India, and Thailand from a murine rodent of Thailand, *Bandicota indica* [10,45]. Recently, new hantaviruses have been isolated from different Rattus spp., during a study conducted in agricultural and urban areas in Cambodia [33]. A phylogenetic analysis of the partial S sequences of these viruses showed that viruses isolated either from *Rattus rattus* or *Rattus norvegicus* could be grouped into two different clades. During this last study, 75 specimens of *Bandicota* sp. were also analyzed and were found to be negative. All these results suggest that different types of hantaviruses are present in South Asia. Thus, extended investigations have to be completed to check if *B. indica* is the specific (and perhaps the single) natural host of Thailand virus; the biodiversity of closely related viruses present in other rodents living in the same geographic area; the phylogenetic relationships of these newly discovered viruses; their respective geographic distribution and if they may be considered a danger for humans living in the same area.

16.2.3 Morphology

The Bunyaviridae family to which genus hantavirus belongs are enveloped viruses with a genome that consists of three negative-sensed single-stranded RNA segments [1,9,37,38,39]:

- A small genomic segment S (1.8 kb) encoding the nucleocapsid N protein.
- A medium genomic segment M (3.7 kb) encoding a polyprotein that is cleaved to yield the envelope glycoproteins G1 and G2.
- A large genomic segment L (6.5 kb) encoding the L protein, which functions as the viral transcriptase replicase.

They include three structural proteins: two glycoproteins G1 and G2, and a nucleoprotein N. Several serologically distinct groups, associated with different syndromes, have been recognized: in the "Old World," Hantaan, Dobrava, Seoul, and Puumala cause the clinical forms of HFRS, whereas, in the "New World," Sin Nombre and Andes are responsible for HPS [15,38]. Another group, Tula, widely distributed in Russia and Eastern Europe, to central Asia and Siberia, has never been associated with any human disease [30].

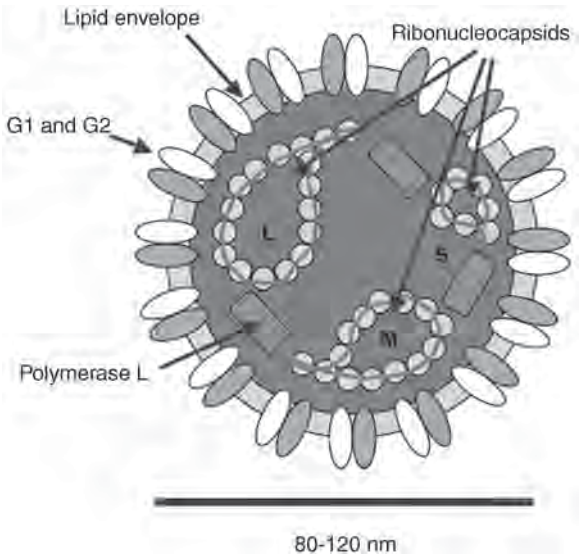

Fig. 16.1. Bunyaviridae virion structure. The viral genome is composed of three ssRNA segments: one large (L) segment, one medium (M) segment, and one small (S) segment. All three segments have the same complementary sequences at the 5′ or 3′ termini.

16.2.4 Transmission

Unlike other members of the Bunyaviridae family hosted by mosquitoes, ticks or flies, specific wild rodent hosts, from the family Muridae, usually carry hantaviruses. These rodents shed the virus in their urine, feces, and saliva. Tiny droplets containing the virus get into the air: "aerosolization." Potentially pathogenic for humans, hantavirus infection occurs through inhalation of virus-contaminated aerosols of rodent excreta. Rodents are therefore the reservoir host for hantaviruses; infections can be spread among the natural hosts by aerosols and bites. As the virus is found in rodent saliva, feces, and urine, humans can become incidental hosts when they come into contact with infected rodents or their excretions. Often, rodent urine, droppings, or nests are disturbed in enclosed areas; the viruses are then inhaled in aerosolized dust. Hantaviruses can also be transmitted through broken skin, the conjunctiva, and other mucous membranes, by rodent bites and possibly by ingestion of contaminated food. Arthropod vectors do not seem to exist. Vertical transmission also appears to be negligible or nonexistent. Person-to-person spread has not been seen in HPS cases in North America or HFRS in Eurasia but may occur with the Andes virus in Argentina. Hantaviruses are sensitive to drying but have been found in neutral solutions for several hours at 37 °C and for several days in colder temperatures. Infectious viruses have also been detected in dried cell cultures for up to 2 days. Hantavirus spp. responsible for HFRS are closely associated with Murinae and Arvicolinae rodents. Sigmodontinae and Neotominae rodents transmit those responsible for HPS in the New World. They cause persistent asymptomatic infections in their natural hosts.

16.2.5 Diagnosis and Symptoms

Although hantaviral infection has been recorded worldwide, cases are not well reported. The incubation period varies from 14 to 17 days; most often, the symptoms appear after 14–30 days. Initial onset is marked by nonspecific flu-like symptoms, leading to diagnostic confusions with other common fevers, especially in the tropics: fever, chills, myaglia (muscle aches), headache, malaise, abdominal pain, nausea, vomiting dry, cough, or tachypnea (increased respiratory rate). In severe HFRS cases, symptoms include hypotension, shock, respiratory failure, and renal impairment or failure. In severe HPS cases, complications can cause cardiorespiratory failure. Most of the patients infected with Sin Nombre virus were reported to die after a few days. Hantavirus outbreaks are often associated with increased rodent populations or environmental factors that lead to increased human exposure to rodents. Worldwide, approximately 150,000 to 200,000 (excluding China) patients are hospitalized with HFRS each year. Different hantaviruses tend to cause mild, moderate, or severe cases of HFRS; the mortality rate can vary from 0.1% to 3% for Puumala virus infections, to approximately 5% to 15% for Hantaan and Dobrava virus infections. Seoul virus tends to cause moderate disease with mortality rates of approximately 1%. Sin Nombre and New York virus infections are often fatal; the mortality rate is estimated to be 40–50%. The renal variant form of HPS caused by the Andes, Bayou, and Black Creek viruses also has a high mortality rate. Convalescence from either HFRS or HPS can take weeks or months, but patients usually recover full lung function.

16.3 SEROLOGICAL PRESENCE WITHOUT CASES IN THAILAND

Rodents are a highly successful group of mammals occurring throughout the world in a wide variety of ecosystems. Although most rodent species live in the wild with little human interaction, some have adapted to human presence and activities, using agriculture and waste as food resources and nesting in buildings. They are considerable agricultural pests destroying crops or food stocks. In Asia, rodents cause 5–10% production loss of rice, which would feed 200 million Asians for a year (CSIRO). Rodents are also important reservoirs and vectors of organisms, which can spread in fields or habitations and cause more than 60 known diseases in humans and livestock. The close proximity between rodents and humans or animals favors the transmission dynamics of these diseases.

Considering the public health importance of rodents with the emergence of leptospirosis in 1996, the Ministry of Public Health and different research institutes has conducted trapping expeditions over the country. Most investigations have focused on serological surveys of leptospirosis or rickettsial diseases. Since 1998, the French Institute of Research Development (IRD) has sampled rodents in different biotopes representative of the rich biodiversity, focusing on their diversity and ecology, between January 1998 and December 2004. One thousand seven hundred and eighteen murine rodents, belonging to 30 different species, were trapped. A new identification field key for the Thai murine rodents was realized. Field campaigns were conducted closely with local farmers to exchange knowledge on rodent ecology and set up the trapping. Throughout the country, some people hunting and eating rodents regularly, showed a real knowledge of the different species. Representing a great part in the biomass, rodents are considered as a valuable source of meat. Hunters can recognize their burrows, noise and attract them with proper baits, chosen for their attractiveness in each place.

Rodents are trapped live and identified by species. Live trapping allows simultaneous monitoring of sympatric populations (species co-occurring in a same ecosystem). Methods can

Fig. 16.2. *Bandicota savilei*, a vector of Hantaviruses in Southeast Asia.

Fig. 16.3. Farmer demonstrating a traditional trap in Phrae province, northern Thailand.

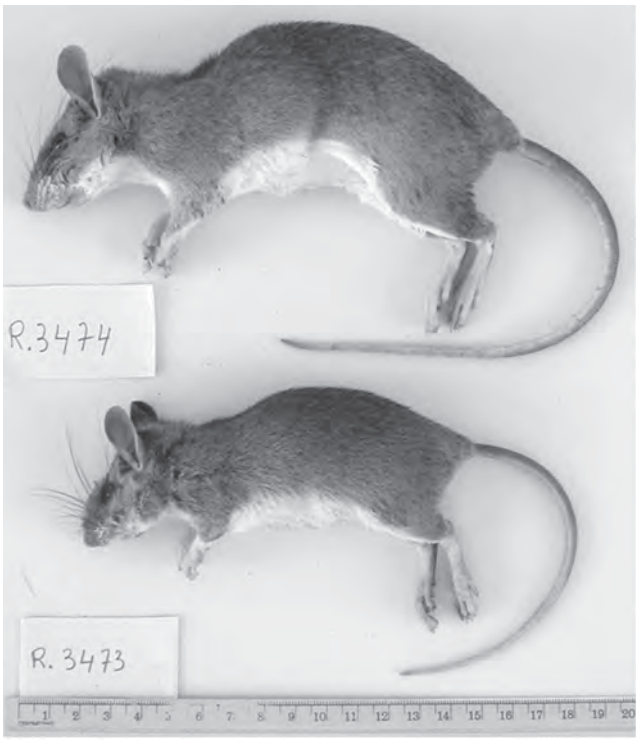

Fig. 16.4. Dealing with the difficulties of morphological identification: two subspecies of *Maxomys surifer* from Loei province, northeastern Thailand.

vary according to logistic and other priorities. Information is recorded on the sex and morphology: head–body length, tail length, skull length, ear length, feet length, and weight. Each place of capture is geographically referenced and described regarding the ecological characteristics. Blood samples are obtained from cardiac puncture immediately after the animal has been euphonized under strong ether anesthesia. Tissue specimens (lung, liver, spleen, and kidney) are collected and transferred immediately into liquid nitrogen. After processing, each animal voucher specimen is placed into 80% ethanol and labeled. Tubes are transported in liquid nitrogen to laboratories in Bangkok for later analysis of viral antibodies.

Fig. 16.5. Dissection in a laboratory at the Ministry of Public Health, in Sakhon Nakhon province, northeastern Thailand (Dr. Vincent Herbreteau).

16.3.1 Serological Investigations in Rodents

First serological investigations of hantaviruses in rodents were conducted between 1981 and 1983, in the frame of a worldwide survey of hantaan-related viruses. A global 7% (21/311) seropositivity was recorded in Thailand, with the highest prevalence, 15% (10/65), for *B. indica*, for the first time recognized as a major vector in Southeast Asia. Neutralization tests, which detected antibody in Rattus specific for hantaan-related viruses, failed to establish the specificity of antibody in *B. indica*, suggesting the occurrence of another hantaan-like virus in Thailand. High prevalence for *B. indica* was reported again in Kanchanaburi province in 1985 with 28.6% positive by IFA [10]. Then, in 1989, serological surveys in northern Thailand reported 4% seropositivity for *R. norvegicus* and 13% for *R. rattus* [35]. In 1990, another investigation in two Bangkok slum areas found one-third of *R. norvegicus* (19/61) and 6% of *Rattus exulans* (1/17) had hantavirus reacting sera [42]. In 1992, 110 rodents from Chiang Rai (northern Thailand) were tested by IFA (Leitmeyer, personal communication) for evidence of antibodies or antigens to four hantaviruses. Antigen-positive specimens were tested by RT-PCR for viral nucleic acids but first attempts of virus isolation failed. *B. indica* appeared as a main vector in rural areas with a high seropositivity 23.5% (8/34). Surprisingly, one *Mus cervicolor*, an endemic rare mouse of rice fields, tested positive to Hantaan virus [42]. Between 1998 and 2000, from 862 murine rodents collected throughout the country, 2.9% tested positive by immunofluorescent assay (IFA). In 1998, investigations on 692 rodents from Nakhon Pathom and Nakhon Ratchasima revealed lower prevalence to hantavirus reacting antibodies when tested by ELISA, which is a more specific method than IFA: 4.9% (16/325) of *R. exulans*, 4.6% (8/175) of *B. indica*, and 4.1% (2/49) of *R. norvegicus* tested positive [27]. In 2002, similar antibody prevalence for *B. indica* (4.3%) but lower for *R. exulans* (2.1%), *R. rattus* (0.9%), and *R. losea* (1.6%) were obtained by ELISA while surveying rodents from five provinces in northeastern Thailand [27].

During these surveys, positive samples could be found in the different regions, North, Northeast, Central Plain, and South, suggesting a wide distribution of hantaviruses in Thailand [27]. Serological surveys could identify hantavirus reacting antibodies in the most common species, also the easiest to catch: *B. indica*, *B. savilei*, *R. rattus*, *R. norvegicus*, *R. losea*, *R. exulans*, and *M. cervicolor* (Table 16.2). Species, which tested negative, *Berylmys berdmorei*, *Mus caroli*, and *Mus castaneus*, were not collected in sufficient number for statistical significance. Only *R. argentiventer*, with 77 specimens tested negative, seems not to be a host of hantavirus. Other murine rodents can be potential hosts or vectors of hantavirus. No significant differences in hantavirus antibody prevalence were found between males and females [27]. Knowledge of prevalence of hantavirus in rodents is limited by the difficulties of field sampling

TABLE 16.2. Serological Investigations of Murine Rodents in Thailand, Tested for Hantavirus Reacting Antibodies

Species	Location	Seropositivity[a]	Test	Reference
Rattus norvegicus	Bangkok (C, 15)	19/61 (31.1) 7/458 (1.53)	IFA 32-128	[42] Kantakamalakul et al., 2003
	Nakhon Pathom (C, 14)	2/49 (4.1)	ELISA	Gonzalez et al., 1998, unpub.
	Chonburi (E, 16)	0/25	PA 1:80	Imvithaya et al., 2001
	Khon Kaen (NE, 6)	0/35	ELISA	Nitatpattana et al., 2002
Rattus exulans	Bangkok (C, 15)	1/17 (5.9)	IFA 32-128	[42]
	Nakhon Pathom (C, 14)	1/45 (2.2)	ELISA	[27]
	Nakhon Ratchasima (NE, 9)	9/257 (3.5)	ELISA	[27]
	Trang (S, 19)	1/26 (3.8)	PA 1:80	Imvithaya et al., 2001
	Udon Thani (NE, 3)	0/25	PA 1:80	Imvithaya et al., 2001
	Surin (NE, 11)	1/49 (2.0)	ELISA	Nitatpattana et al., 2002
	Nakhon Phanom (NE, 5)	1/31 (3.2)	ELISA	Nitatpattana et al., 2002
	Kanchanaburi (C, 12)	0/102	ELISA	Herbreteau et al., 2003, unpub.
	Sakhon Nakhon (NE, 4)	0/19	PCR	Herbreteau et al., 2003, unpub.
Rattus rattus	Chiang Rai (N, 1)	3/50 (6)	IFA 32-2048	Leitmeyer, 1996, pers. comm.
	Nakhon Pathom (C, 14)	1/68 (1.5)	ELISA	[27]
	Nakhon Ratchasima (NE, 9)	0/36	ELISA	[27]
	Surat Thani (S, 18)	2/40 (5.0)	PA 1:80	Imvithaya et al., 2001
	Phra Nakhon Sri Ayuthaya (C, 13)	1/67 (1.5)	PA 1:80	Imvithaya et al., 2001
	Chonburi (E, 16)	3/30 (10.0)	PA 1:80	Imvithaya et al., 2001
	Phitsanulok (N, 2)	3/88 (3.4)	PA 1:80	Imvithaya et al., 2001
	Chantaburi (E, 17)	0/83	PA 1:80	Imvithaya et al., 2001
	Trang (S, 19)	1/51 (2.0)	PA 1:80	Imvithaya et al., 2001
	Nakhon Phanom (NE, 5)	1/37 (2.7)	ELISA	Nitatpattana et al., 2002
	Kanchanaburi (C, 12)	1/43 (2.3)	PCR	Herbreteau et al., 2003, unpub.
Rattus losea	Chiang Rai (N, 1)	6/25 (24.0)	IFA 32-2048	Leitmeyer, 1996, pers. comm.
	Buriram (NE, 10)	0/26	ELISA	Nitatpattana et al., 2002
Bandicota indica	throughout country	10/65 (15)	IFA 32	LeDuc et al., 1986
	Chiang Rai (N, 1)	8/34 (23.5)	IFA 32-2048	Leitmeyer, 1996, pers. comm.
	Nakhon Pathom (C, 14)	4/151 (2.6)	ELISA	Gonzalez et al., 1998, unpub.
	Nakhon Ratchasima (NE, 9)	4/24 (16.7)	ELISA	Gonzalez et al., 1998, unpub.
	Petchabun (C, 6)	3/49 (6.1)	PA 1:80	Imvithaya et al., 2001
	Phitsanulok (N, 2)	2/52 (3.8)	PA 1:80	Imvithaya et al., 2001
	Nakhon Ratchasima (NE, 9)	2/53 (3.8)	PA 1:80	Imvithaya et al., 2001
	Nakhon Phanom (NE, 5)	0/59	ELISA	Nitatpattana et al., 2002
	Khon Kaen (NE, 7)	6/49 (12.2)	ELISA	Nitatpattana et al., 2002
	Buriram (NE, 10)	2/37 (5.4)	ELISA	Nitatpattana et al., 2002
	Surin (NE, 11)	1/25 (4.0)	ELISA	Nitatpattana et al., 2002
	Kalasin (NE, 8)	0/38	ELISA	Nitatpattana et al., 2002
	Kanchanaburi (C, 12)	0/16	PCR	Herbreteau et al., 2003, unpub.
Bandicota savilei	Nakhon Pathom (C, 14)	0/13	ELISA	Gonzalez et al., 1998, unpub.
	Nakhon Ratchasima (NE, 9)	0/10	ELISA	Gonzalez et al., 1998, unpub.
	Phra Nakhon Sri Ayuthaya (C, 13)	2/25 (8.0)	PA 1:80	Imvithaya et al., 2001
	Chonburi (E, 16)	0/22	PA 1:80	Imvithaya et al., 2001
	Phitsanulok (N, 2)	0/36	PA 1:80	Imvithaya et al., 2001
Berylmys berdmorei	Kanchanaburi (C, 12)	0/11	PCR	Herbreteau et al., 2003, unpub.

[a]Number positive/total tested (percentage positive). N, north; NE, northeast; C, central; E, east; S, south. 1 to 19: Province number on Thailand map. PA, particle agglutination test; Unpub., unpublished; pers. comm., personal communication.

to catch rare species, and get knowledge about each species density, but also by the dated taxonomy of murine rodents in Thailand, actually under revision.

Serological investigations of murine rodents in Thailand, tested for hantavirus reacting antibodies.

A recent study was conducted in agricultural and urban areas in Cambodia to assess the presence of hantaviruses in rodent populations [33]. In 1998, rodents were trapped in two villages and in Phnom Penh city near market places and a rubbish dump. IgG antibodies to Hantaan virus were detected in

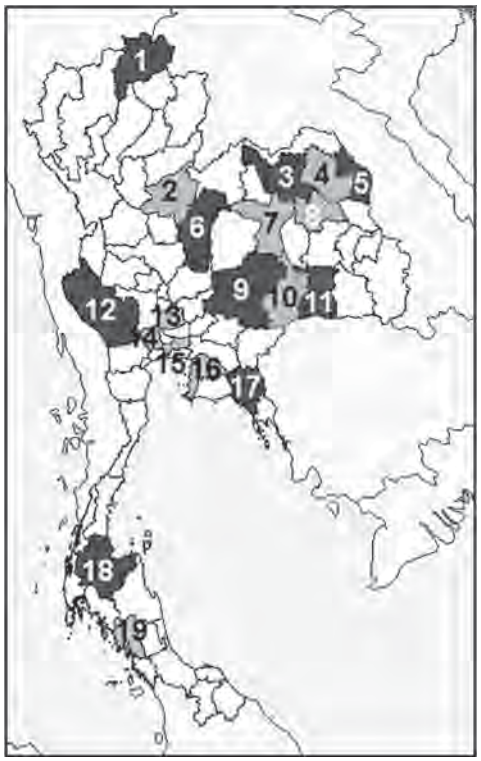

Fig. 16.6. Location of Thai provinces where murine rodents were tested for Hantavirus-reacting antibodies.

54 (8.2%) rodents among 660 tested: 6.4% (13/203) among *R. rattus*, 20.9% (39/187) among *R. norvegicus*, 16.7% (2/12) among unidentified Rattus species, and none in 183 *R. exulans* or in 75 Bandicota spp. The presence of the viral genome was detected by a reverse transcription PCR amplifying part of the sequence coding for the nucleoprotein in the S segment, in 87% of the seropositive rodents. Thirty-one representative cDNAs were sequenced. Phylogenetic studies of the sequences indicated a close relationship with Seoul virus. However, the Cambodian-Seoul virus strain sequences clustered within two different phylogenetic lineages, one associated with *R. rattus* and the other with *R. norvegicus*.

16.3.2 Serological Investigations in Humans

Serological surveys carried out to detect evidence of hantavirus infection in human populations revealed that in Thailand, in different provinces and/or in different environments, 1.2–31.4% of individuals tested had hantavirus antibody [27,35]; the recent publication of the first human case in Thailand confirms the presence of hantavirus in Southeast Asia [40].

All this suggests that rodents are probably the primary reservoir, and that other mammals may be involved in the cycle of hantaviruses; new viruses, different hosts and different human syndromes may be expected to be discovered in the future. Additional work is needed in the traditional areas where hantaviruses have been recorded or suspected, mainly in Southeastern Asia where murine rodents are present, highly diversified and certainly reservoirs for hantaviruses.

16.4 PHYLOGENY OF HANTAVIRUSES

Different analyses, based on alignment of M or S sequences [7,13,16,18,19,20,21,25] have been performed and used to discuss the distribution of the hantaviruses, in relation to the biogeography and evolutionary history of their hosts. Generally, these studies were based on mixed data sets including sequences issued from wild mammals (collected in their natural range), and sequences exclusively known from human patients. Also, most often they were based on neighbor-joining analyses and incomplete data sets (including only a part of the known diversity of the viruses among their natural hosts), or data sets limited to particular geographic areas. The strong growth of phylogenetic biology during last two decades has been aided by recognition of the importance of a correct phylogenetic analysis as a necessary step, before interpreting evolution. Thus, in the following we redo an analysis of the S sequences and use the resulting cladogram to discuss the origin and distribution of rodent-borne hantaviruses.

16.4.1 Material and Methods

16.4.1.1 Sequences alignment Only S sequences found in GenBank of virus isolated from precisely identified wild mammals, including complete CDS, were held. The data set includes 93 taxa (Table 16.1): 91 isolated from different rodent hosts; one isolated in Korea from a bat (Kim, direct submission 1995); Thottapalayam detected in India from a shrew (*Suncus murinus*) by Carey et al. [4], identified by Xiao et al. [45], complete S sequence recently introduced in GenBank by Schmaljohn and Toney (direct submission, 2004) used as outgroup. Retained sequences ranged between 1130 and 2082 nucleotides from which first 42 (primer) and nucleotides 1342–2082 (codon stop and noncoding region) were eliminated; nucleotides 43–1341 (coding part) were used for cladistic analyses. Alignment performed at amino acid level and analyzed at nucleotide level, using CLUSTAL-X [44] and SE-AL v2.0a11 [32].

16.4.1.2 Aligning and coding indels During sequence alignment, it became necessary to include several gaps between nucleotides 766 and 813 (Fig. 16.7). Therefore, more than one equally optimal alignment might be proposed for this region. Comparative secondary structure alignment, currently considered a powerful method [14,28], could not be used here because no model is available for these organisms. We applied Barriel's method [2] of successive parsimony analysis using PAUP* 4.0b10 [41] to test different alignments, produced manually using SE-AL. In order to define the most parsimonious, we used the following criteria: (1) minimize the number of inferred mutations (number of steps), (2) test number of weighed mutations (one transition [Ts], preferred to one transversion [Tv]), and (3) minimize the number of variable sites.

Standard procedures for coding gaps suffer from several weaknesses: either the different sites are analyzed independently (gap = new state) and each gap is artificially weighed in relation

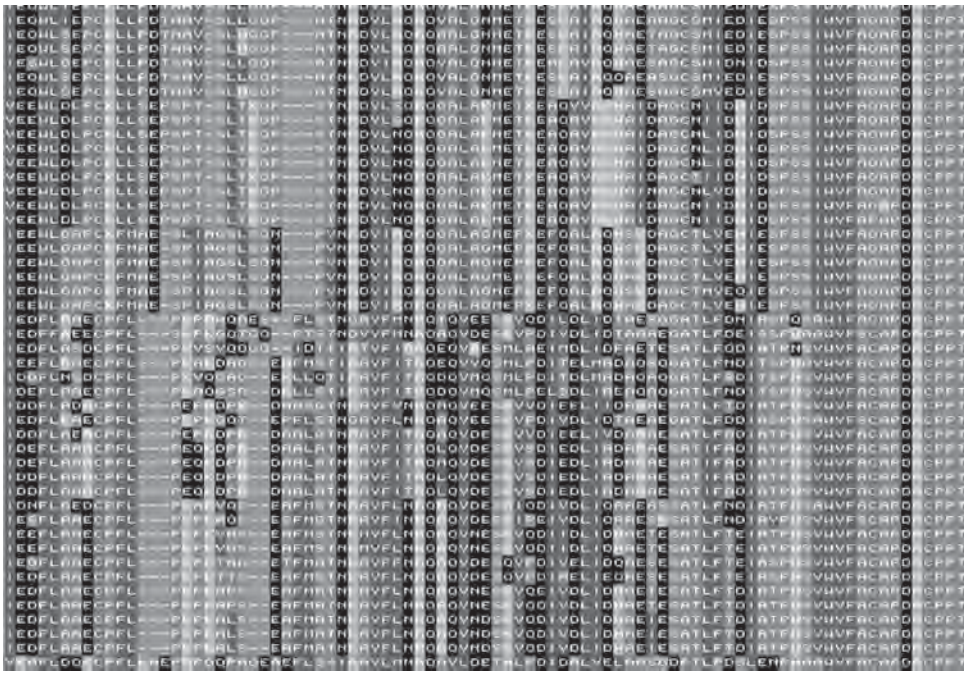

Fig. 16.7. Alignment of the S sequences in the hypervariable (HV) region. The alignment at the amino acid level makes necessary to introduce several indels. The HV region is flanked by two conserved cysteine.

to the number of sites, or each site is coded "?" (gap = missing data) and optimization procedure makes the whole zone devoid of phylogenetic information. To express potential phylogenetic information contained in zones with inter-nested insertions/deletions and substitutions, nine characters coding the presence/absence of deletions between nucleotides 766 and 813 were added. Finally, the matrix includes 1323 RNA characters and nine presence/absence characters.

16.4.1.3 Sequence analyses Two methods likely to give results interpretable in an evolutionary context were used: maximum parsimony analysis (MP) and Bayesian analysis (MB). MacClade 4.0 [23] and Treeview 1.3 [29] were used for data and tree handling and for computation of statistics. MP analysis was computed using PAUP. Robustness of nodes was assessed using bootstrap method [11], computed after 10,000 replicates of heuristic search with closest stepwise addition of taxa. Modeltest 3.0 [31] was used to determine the best fitting settings: the general time reversible model [47] with among-site substitution rate heterogeneity described by a gamma distribution with eight categories [46] and a fraction of sites (INV) constrained to be invariable (GTR+I+G, selected by AIC). MB analysis using these settings was performed using MrBayes v3.0B4 [17]. This approach evaluates the posterior probability of a tree given the character matrix, that is, the probability that the tree is correct. Posterior probability is obtained after combining the prior probabilities of a tree and of the data with the likelihood of the data given that tree. Bayesian approach allows defining an explicit probability model of character evolution and obtaining a rapid approximation of posterior probabilities of trees, through the use of the Markov Chain Monte Carlo (MCMC) approach. MrBayes also allows performing phylogenetic analyses of data sets combining information from different subsets, evolving under different stochastic evolutionary models. Two partitions were distinguished in our original data set: partition 1 = nucleotide (characters 1–1299) for which the likelihood model chosen was the GTR+I+G; partition 2 = indels (characters 1300–1308) treated as presence/absence. Analysis was conducted with four independent Markov chains, run for 500,000 metropolis-coupled MCMC generations, with tree sampling every 10 generations and burn-in after 3300 trees. Consensus tree was computed using the "halfcompat" option, equivalent of 50% majority rule. Proportion values of posterior probability of bipartition, considered equivalent to bootstrap values [6] were used for evaluation of robustness of the nodes.

16.4.2 Results

MP or MB analyses yield consistent results. All bipartitions found by MP analysis with a bootstrap value superior or equal to 95% were also found by MB analysis with a posterior probability equal or superior to 95%. In addition, MB analysis gave a resolution and a support superior or equal to 50% for several nodes, which were unresolved, or resolved with a bootstrap inferior to 50%, in the MP analysis. Even if MB analysis is likely to favor higher values when compared to bootstrap analysis [6,17,48], the results are fully congruent and are presented in Figure 16.8. Figures 16.9 and 16.10 detail the composition of

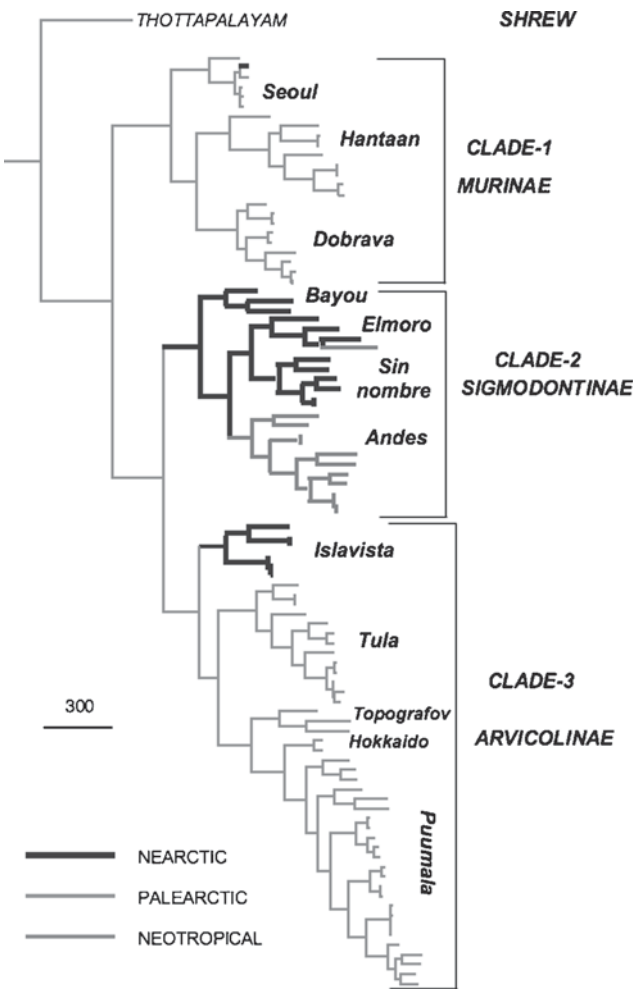

Fig. 16.8. Phylogram resulting from Bayesian analysis using GTR + I + G model. Different color patterns are attributed to different biogeographical areas. Three main clades may be recognized. CLADE-1 and CLADE-2 are detailed in Figure 16.9. CLADE-3 is detailed in Figure 16.10. See color plates.

the three main identified clades. Figure 16.11 summarizes the relation between the virus phylogeny and the host taxonomy.

The cladogram is rooted between a basal branch corresponding with Thottapalayam and a monophyletic group including all the rodent-borne parasites, distributed following three main clades: CLADE-1 includes "Seoul, Hantaan, Dobrava"; CLADE-2 and CLADE-3 are sister clades including "Bayou, Sinnombre, Andes," and "Islavista, Tula, Puumala," respectively. Each clade and the sister grouping of CLADE-2 and CLADE-3 have a support superior or equal to 78%. CLADE-1 groups 22 taxa: all the viruses hosted by Murinae rodents, and the single strain found on a bat; CLADE-2 groups 23 taxa: all the viruses hosted by Sigmodontinae rodents; CLADE-3, groups 48 taxa: all the viruses hosted by Arvicolinae rodents. Regarding the biogeographical distribution, CLADE-1 is exclusively Palearctic, except Tchoupitoulas collected in the Nearctic (Louisiana); CLADE-2 is found exclusively in the "New World" and associates strains from the Nearctic and Neotropics; CLADE-3 may be divided into one Nearctic subclade (Islavista) and the sister grouping of two Palearctic subclades (Tula + Puumala).

16.4.2.1 CLADE-1: "Seoul, Hantaan, Dobrava" (Fig. 16.9) Viruses hosted by Rattus spp. are distinguished from those hosted by *Niviventer confucianus* and Apodemus spp. With the exception of the parasite of Niviventer (considered by taxonomists closer to Rattus), this distribution matches the taxonomy of the rodents at genus level. However, different virus strains hosted by the same rodent species are not grouped together. The bat virus is included in Hantaan; its closest relative is HTNV-76118. Regarding the geographic distribution: Seoul is found in eastern China, with the exception of SEOV-Sapporo (Japan) and SEOV-Tchoupitoulas (Louisiana), which are sister taxa. Hantaan also is restricted to the eastern part of the Palearctic region, but with a wider distribution including several provinces in

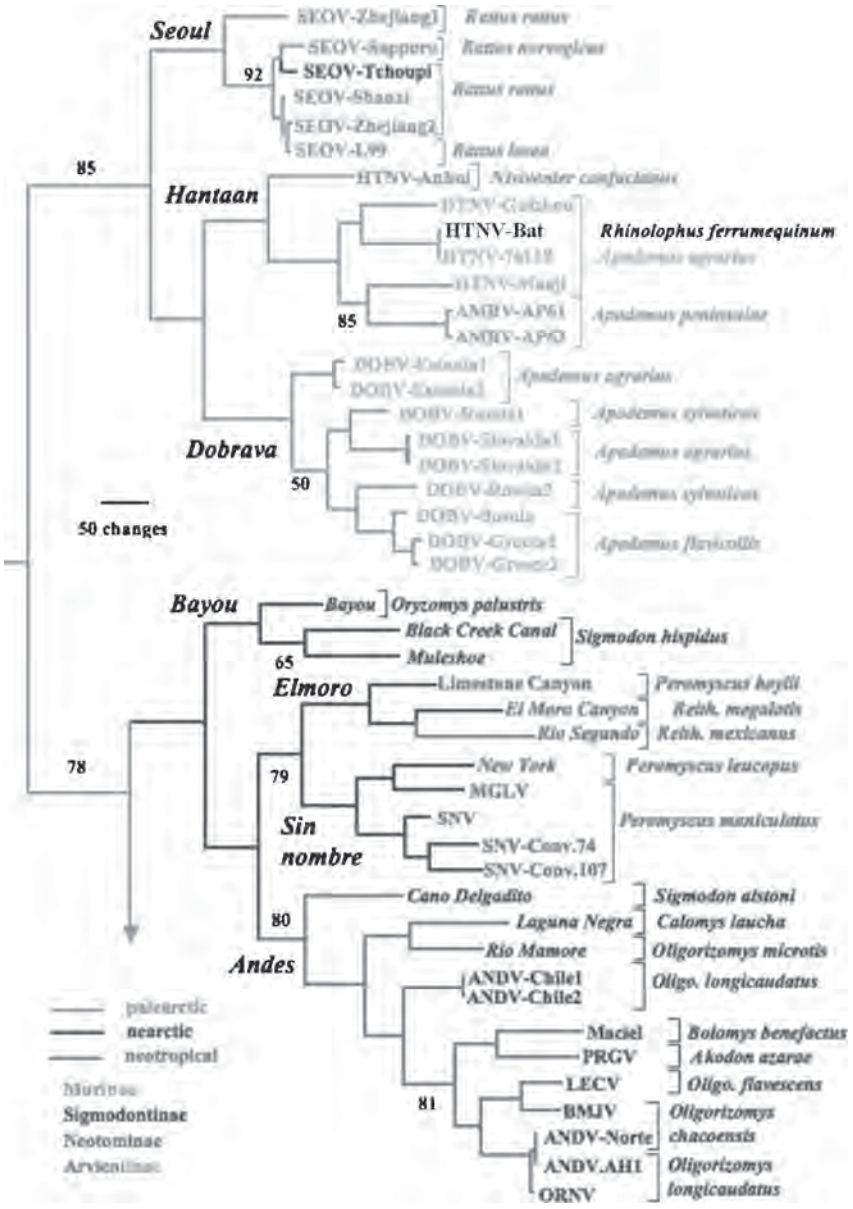

Fig. 16.9. Detail of CLADE-1 and CLADE-2 of Figure 16.8. Posterior probability numbered when inferior to 95% (probability of no numbered nodes between 95 and 100). The scientific name of host for each virus strain is given; different color patterns are attributed to different host groups and to different biogeographical areas. Reith., Reithrodontomys; Oligo., Oligorizomys. See color plates.

China, Korea, and the Amur area (northeastern Siberia). Dobrava has a European distribution extending from Estonia towards Greece, through Western Russia, Slovakia, and Bosnia. The arrangement of Dobrava viruses on the cladogram generally fit with a north to south distribution.

16.4.2.2 CLADE-2: Bayou, SinNombre, Andes (Fig. 16.9) From the three subclades, two are hosted by Sigmondontini rodents (Bayou, Andes), whereas Sinnombre is hosted by Neotomini rodents. Bayou, found in three states of Southeastern North America (Florida, Louisiana, and Texas) is hosted by two different genera, Oryzomys and Sigmodon. Sinnombre is subdivided into a group of three taxa found in Arizona, New Mexico, and Costa Rica, and is hosted by Peromyscus sp. and Reithrodontomys spp.; *a group* hosted by Peromyscus spp. ranging from Northeastern to Southwestern and Central United States. Andes, is exclusively found in the Neotropics and hosted by Sigmodontini rodents: Oligoryzomys is the most frequently, together with several other genera (Akodon, Bolomys, Calomys, Sigmodon). The most divergent species in this group is Caño Delgadito from Venezuela; the other species are arranged following their geographical origin: Laguna Negra and Rio Marmore (Bolivia and Paraguay); ANDV-Chile 1 and 2,

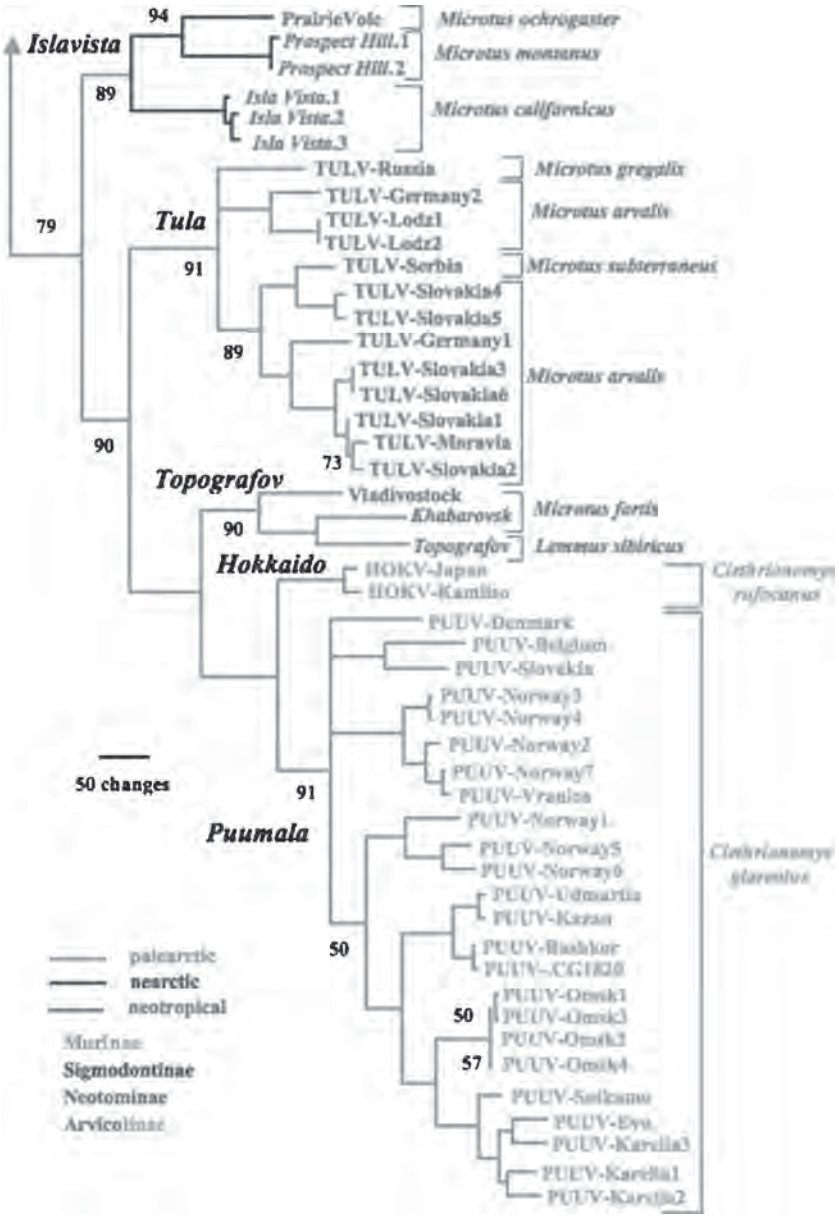

Fig. 16.10. Detail of CLADE-3 given in Figure 16.8. Posterior probability numbered when inferior to 95% (probability of no numbered nodes between 95 and 100). For each virus strain, the scientific name of host is given; different color patterns are attributed to different host groups and to different biogeographical areas. See color plates.

(Chile); the last seven are from Northern Argentina. Distribution of virus taxa within CLADE-2 generally fits with the taxonomy of rodents at host tribe level and a dominant genus may be recognized for each of the main subgroups. However, the Sigmodontini parasites are not monophyletic; as in CLADE-1, no congruence is observed at host species level (closely related viruses hosted by different host species, viruses hosted by a same host species not closely related on the cladogram).

16.4.2.3 CLADE-1: Prairie, Tula, Puumala (Fig. 16.10) CLADE-3 is the sister group of CLADE-2 and is hosted by Arvicolinae rodents. Tula and Puumala are strictly Palearctic, Islavista is strictly Nearctic. Microtus spp. is the dominant host for Islavista and Tula. Islavista may be subdivided into two groups: Islavista 1, 2, 3 are Californian, Prairie Vole and Prospect Hill 1 and 2 are from South Central United States. Tula has a European distribution extending north to south, from Poland, Germany, Moravia, Western Russia, and Slovakia. In Puumala: Microtus, associated with Lemnus, is present in a small basal group including three virus species found in the extreme east of Russian Siberia (Vladivostok, Khabarovsk, and Topografov); the other species are hosted by *Clethrionomys rufocanus* or *C. glareolus*. The parasites of *C. rufocanus* are Japanese strains (Hokkaido). The parasites of *C. glareolus* have a distribution extending from

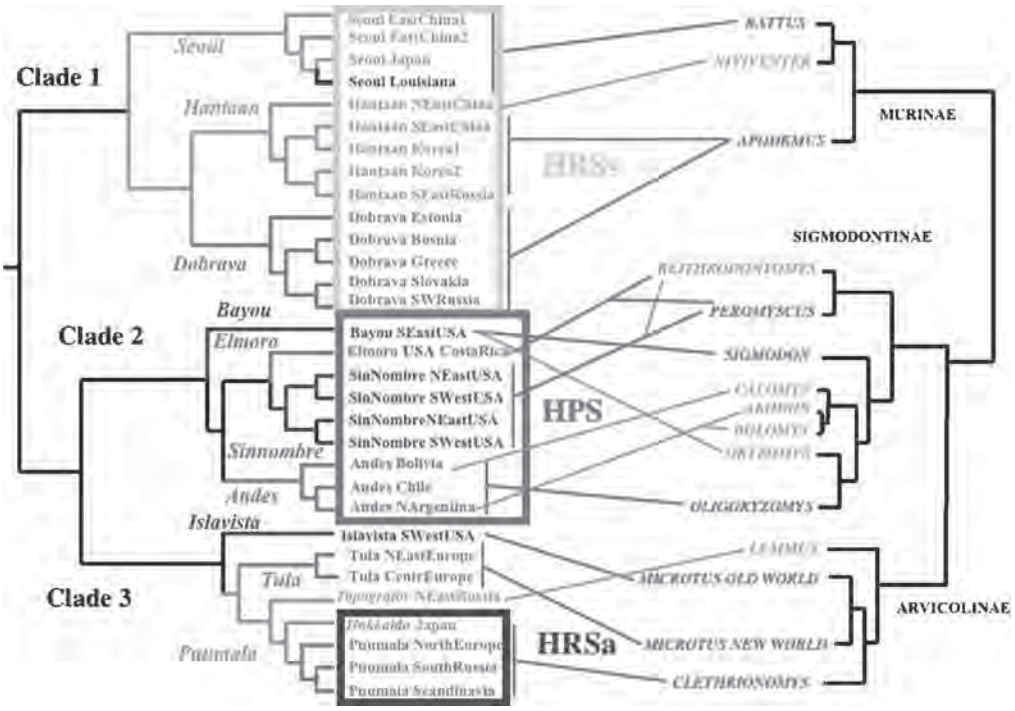

Fig. 16.11. Correspondence between the phylogeny of genus Hantavirus, the classification of its hosts, and the type of human syndrome. See color plates.

Northwestern Europe (Denmark, Belgium) to Scandinavia, Finland, and South Central Russia. Among Puumala, a dominant host species may be recognized for each of the main subgroups. But, in Islavista and Tula, there is no general congruence between virus and host classifications at species level: closely related viruses hosted by different host species; viruses hosted by a same host species, not closely related on the cladogram.

Fig. 16.12. *Maxomys Surifer* kept in a cage, before being sold in a rural market in Phrae province, northern Thailand.

16.5 DISCUSSION

16.5.1 Clades, Groups, Robustness of Nodes, and Molecular Data

Our analysis confirms the three main clades previously described within the hantaviruses [18,25] and supports the subdivision of each clade into three subclades. "Seoul," "Hantaan," "Dobrava," "Andes," "Tula," "Puumala," already have been named. We propose new names for several new groups:

- "Bayou," including Bayou, Black Creek, Muleshoe.
- "Sinnombre," including Sin Nombre, Convict Creek, Monongahela, and New York.

Fig. 16.13. Painting in Wat Wang Luang, in Phrae province, northern Thailand.

- "Elmoro," including El Moro Canyon, Rio Segundo, and Limestone.
- "Islavista," including Prairie Vole, Prospect Hill, and Isla Vista.
- "Topografov," including Topografov, Khabarovsk, and Vladivostock.
- "Hokkaido," including Hokkaido and Kamiiso-8cr-95.

The support for corresponding nodes of the cladogram is generally between 80 and 100. The alignment shows that main clades and subclades are supported by amino acid changes caused by synonymous or non-synonymous nucleotide differences. Most changes occur in the HV region (Fig. 16.7) identified by several previous studies [22,30]. Hughes and Friedman [18] defined the HV region as residues 242–281. In our joint alignment, HV region corresponds to amino acid residues 249–317 and includes 92% of informative sites (for whole matrix, the percentage is 62%). This region also includes several regular indels corresponding with the main subdivisions of the cladogram.

16.5.2 Biogeography of Hantaviruses and Their Hosts

16.5.2.1 Host specificity and correspondence with host taxonomy Lundkvist et al. [22], observed that "···evidence that at least in some hantaviruses the HV region is a target for host antibodies and · · · known importance of charged residues in determining antibody epitopes · · · suggest that changes in the HV region may represent adaptation to host-specific characteristics of the immune response." The strong correspondence between the indels and variations in the HV region and the distribution of the hantavirus in identifiable rodent groups support this hypothesis (Fig. 16.7).

The topology of the three main clades matches the phylogeny of the three host subfamilies to which they are respectively devolved. Within CLADE-1 and CLADE-3 different subclades have a dominant host genus easily recognizable. Within CLADE-2, a particular host tribe hosts each subclade, but it is less easy to identify a dominant genus (Fig. 16.8). The good correspondence of the phylogenies at their highest level is consistent with the hypothesis of coevolution: the hantavirus and the Muridae may have evolved and dispersed in parallel. But, whatever the clade considered, there is a mismatch of the host and parasite distributions at species level. It appears as if the host specificity disappeared somewhere between the species and/or genus level. Depending on the clade considered, this limit is variable: host switching at genus level appears difficult and unlikely within CLADE-1 and CLADE-3, and easier in CLADE-2; within CLADE-2, the highest diversity, thus weakest specificity at genus level, is observed in Andes.

16.5.2.2 Biogeography of rodent-borne hantaviruses CLADE-1 is Palearctic except Tchoupitoulas, reported from a wild *R. norvegicus* in New Orleans. *R. norvegicus* is a cosmopolitan species, whose dependence on human living areas is well known and the presence of this hantavirus in the "New World" can be, interpreted as a case of dispersion by humans. CLADE-2 is exclusively found in the "New World": Figure 16.2 shows that unexpectedly following the hypothesis of coevolution, the parasites of the Nearctic Sigmodontini (Bayou) are not closely related to the parasites of Neotropical Sigmodontini (Andes). Most of the Sigmodontini biodiversity is found in the Neotropics, whereas their sister group, the Neotomini, is dominant in North America. Bayou seems limited to Southeastern United States, and may perhaps be interpreted as resulting from an ancient isolation of its hosts in a remote part of their range. CLADE-3 has a mixed distribution with one small Nearctic subclade (Islavista) and four Palearctic subclades (Tula, Topografov, Hokkaido, and Puumala). Islavista, Tula, and Topografov are hosted by different species of genus Microtus, Puumala, and Hokkaido are hosted by Clethrionomys spp. This distribution is consistent with a Palearctic origin, a passage into the "New World" probably transported by the Arvicolinae (most probably Microtus), a later dispersion in North and South America following the migrations of the Sigmodontinae. The usual hypothesis generally accepted for the radiation of Muridae is that of starting from their South Asian center of origin and having a parallel evolution. Within the subclades a different pattern is suggested, because transmission between different rodent species in a same genus (and between different genera in the Neotropics) looks possible.

Finally, two different patterns of dispersion explain the evolution of hantaviruses: the first one, characterized by a strong specificity for a particular group of hosts, explains the ancient history of this group and its coevolution with Muridae; the second one, characterized by a slack specificity, is corresponding with the recent and current history of viruses and their opportunistic circulation by using contacts between closely related rodent genera, species, and/or populations. This second pattern explains why from the point when host and parasite distribution was well documented (Dobrava, Tula, Puumala, and Andes), a geographic gradient become visible. Different pattern, following different specificity is in agreement with what is known about hantavirus survival outside their hosts. Sauvage et al. [34], considering the role of indirect transmission on virus persistence, suggest that viruses remain active outside the host, which could permit transmission without physical contact of infectious rodents. This explains how hantaviruses may switch when the specific barrier is low and when different hosts have overlapping territories.

16.5.2.3 Comparison with previous studies The subdivisions of our cladogram in recognizable clades and subclades are generally fitting with groups already defined by previously published papers using different genes (S or M), different methods, and different data sets. However, some differences appear within clade arrangement (Fig. 16.11).

Within CLADE-1, following the authors, Dobrava is associated either with Hantaan or Seoul. Within CLADE-2, Limestone, El Moro, Rio Segundo constitute a particular clade, neither included in SINNOMBRE nor associated with another subgroup; Sinnombre and Andes sometimes are sister groups, but sometimes are associated differently. Within CLADE-3, in most phylogenic studies published previously, Tula is the sister group of Islavista.

In our results, the sister grouping of Dobrava with Hantaan is strongly supported (posterior probability superior to 80%) and several characters common to all parasites of Apodemus spp. may be observed in the alignment: a common deletion between nucleotides 784 and 796; several sequences of nucleotides, particularly in the HV region. Thus, our results support the hypothesis of a close relationship between the two main groups parasitizing Apodemus spp. There is a strong case for grouping the triplet Limestone, El Moro, Rio Segundo within a particular clade associated with Sinnombre. This topology associates all the parasites of Peromyscus spp. (in Sinnombre + Elmoro), grouping together taxa which has a particular geographic distribution and suggests that the "New World" hantavirus were first established in the Nearctic and secondarily emigrated to the Neotropics. Finally, within CLADE-3, the opposition of a Nearctic group (Islavista) and a Palearctic group (Tula + Topografov + Hokkaido + Puumala) is also well supported by data (posterior probability ranging between 80 and 90). If we compare our topology with the grouping of Islavista and Tula, both topologies support Microtus spp. as primary hosts, but our topology supposes an earlier separation between the Nearctic and the Palearctic species of this genus. Thus, generally our results support hypotheses in agreement with the most parsimonious interpretation of a parallel evolution of genus hantavirus and the Murinae.

16.5.2.4 What are the limits of the hantavirus range?

Although most Bunyaviridae are hosted by arthropods, genus hantavirus has rodents as principal hosts. However, two strains have been isolated from non-rodent mammals: Thottapalayam, isolated from a shrew; the Hantaan virus isolated from a bat. Thottapalayam sequence possess a common deletion with the members of CLADE-1 between nucleotides 805 and 813, but makes necessary the addition of several deletions when introduced in the alignment and lacks several conservative parts of the rodent-borne sequences. Thus, if Thottapalayam can be considered a hantavirus, it is highly divergent from other members of the genus. This is confirmed by its position in the cladogram and by values of total-character distances calculated using PAUP within the rodent-borne group, distances vary from 2 to 516; between Thottapalayam and others, distances range between 765 and 859. This suggests that Thottapalayam probably does not result from a recent host switching between rodent and shrew. Further investigations are needed to decide if this adaptation to a different group of mammals is incidental, or may represent the emerging tip of a different lineage.

The bat virus is included in Hantaan; its closer relative is HNVT.7611. No significant difference of branch length is observed between the two strains and their total-character distance equals 4, suggesting that the two sequences are almost identical; thus, the presence of a different virus species in *R. ferrumequinum* cannot be considered strongly established.

Most of hantavirus spp. found in wild animals were collected in the Holarctic, or the Neotropics (Northern Asia, Europe, North America, and South America). But, Thottapalayam comes from South Asia, and Thailand virus comes from Southeastern Asia where it is hosted by *B. indica*, a Muridae rodent. Also, serological surveys carried out to detect evidence of hantavirus infection in human populations revealed that in Thailand, in different provinces and/or in different environments, 1.2% to 31.4% of individuals tested had hantavirus antibody [26,35,42,43]; similar screenings, performed in West and Central Africa where human hantavirosis has not yet been reported, show that humans may have been infected by Hantaan-related virus [12]. All this suggests that if rodents are probably the primary reservoir, other mammals may be involved in the cycle of hantaviruses; new viruses, different hosts and different human syndromes may be discovered in the future. Additional work is needed in the traditional areas where hantaviruses have been recorded, mainly in Southeastern Asia and in Africa where Muridae rodents are present and highly diversified.

16.6 CONCLUSION

16.6.1 Presence Without Cases Versus Cases Without Notification?

With proof of hantaviruses presence in different rodent species and proof of regular transmission of rodent-borne diseases to humans for years, questions subsist as for the few notified cases and a unique confirmed one.

The presence of a virus and its vector in the environment does not necessarily imply human cases. The possibility of infection depends on a combination of factors conditioning the vulnerability and exposure of people. First of all, the transmission to humans occurs if people are exposed to the infections, requiring being in close proximity to rodents. These conditions exist in Southeast Asia where rodents are regularly hunted and eaten in the countryside. Even inside habitations all over the country, rats and especially the Polynesian rat, *R. exulans*, is common. In some villages, well-known hunters act as meat seller, keeping animals in cages at home or selling them in fresh markets.

Although culture and regionalism may show different situations of exposure, a global high exposure is expected for rural populations.

Lastly, the absence of cases could reflect a public health system not able to detect them. Even if Thailand is globally covered with public health infrastructures providing low-cost health care, strong inequalities subsist between social classes and regions. In rural areas, recourse to health services occurs

in case of severe fevers, a spontaneous behavior being to take paracetamol. Some cases may not be recorded. Another difficulty remains in the clinical diagnosis and possible confusion with other recurrent fevers in Thailand: leptospirosis, scrub typhus, or even dengue, some cases being also classified as "fever of unknown origin."

ABBREVIATIONS

cDNA:	Copy deoxyribonucleic acid
ELISA:	Enzyme-linked immunosorbent assay
HFRS:	Hemorrhagic fever with renal syndrome
HPS:	Hantavirus pulmonary syndrome
IFA:	Iimmunofluorescence antibody detection test
RNA:	Ribonucleic acid
RT-PCR:	Reverse transcriptase polymerase chain reaction

GLOSSARY

Aerosolization: Conversion into an aerosol. Aerosolization is the process of creating very small droplets of moisture that may carry microorganisms. The aerosolized droplets can be light enough to remain suspended in the air for short periods of time and facilitate inhalation of microorganisms.

Arvicolinae: A subfamily of the family Muridae, comprising at least 143 species. Voles and lemmings are Holarctic. Thus, within the three subfamilies of the Muridae, which are known to host some hantavirus, only the Arvicolinae are present on both sides of the Bering Strait. The Arvicolinae seem to be more comfortable in cold countries, and they are abundant and well adapted to the cold climate of Scandinavia, Siberia, and the most northern part of North America. However, some species are known in the southern part of their range, in Pakistan and India, as well as in Mexico and Guatemala.

Bayesian analysis: Bayesian inference is a statistical inference in which probabilities are interpreted not as frequencies or scale, but rather by their degree of credibility. The name comes from Bayes' theorem, which is frequently employed in this type of analysis.

Clade: (Greek: klados = branch). A clade is a monophyletic group of organisms. The members of a clade are all the organisms sharing one unique common ancestor, and this ancestor itself.

Cladistics: A method of classifying organisms, which requires all taxa to be clades. Recognition of the taxa to be included in a clade is based on the existence of at least one derived similarity, or "shared derived properties," or synapomorphies. Cladistics is opposed to "Phenetics," in which organisms are grouped based on their overall similarity.

Cladogram: Tree-like relationship diagram in which all organisms lie at the leaves, and each inner node represents the common ancestor of the dependent leaves. Ideally, a cladogram is binary. On either side of a split, the two taxa are called sister taxa or sister groups. Each sub-tree is a clade. Each clade is set off by at least one synapomorphy (one shared, derived character).

Hantavirus: (derived from the Hantaan River, where the etiologic agent of Korean hemorrhagic fever, the Hantaan virus, was first isolated) One of the five genera of the family Bunyaviridae, hantaviruses are spread by rodents, transmitted by aerosolization and target the kidneys, lungs or pulmonary system, and heart.

Muridae: The largest family within the mammalians contains over 1300 species, 281 genera, and 17 subfamilies. The origins of the Muridae are believed to be in Southeast Asia. Within this family, three subfamilies are known reservoirs for Hantavirus: Arvicolinae, Murinae, and Sigmodontinae.

Murinae: A subfamily of the family Muridae, comprising at least 423 species in 129 genera. The Murinae are the most successful group within the Rodentia. They have an extended natural range, but are limited to the "Old World": from Africa to Australia, from Europe and Eurasia to Asia. This group includes mice, rats, and their relatives. These species live commensally with humans and have reached a worldwide distribution.

Phylogenetics: (from Greek: phylon = tribe, race; genetikos = relative to birth) The study of evolutionary relatedness among various groups of organisms (e.g., species, populations).

Phylogeny: The evolutionary relationships between different species of organisms as represented in a phylogenetic tree. In molecular phylogeny, these relationships are determined by analysis of the similarities and differences in the sequences of genes common to various species.

Reservoir or carrier: A person or an animal, in which an infectious agent lives, which may not itself have any visible signs of disease caused by carrying the agent. The carrier can transmit the disease to humans or animals. Host may also be used.

Sigmodontinae: They are the second-largest subfamily of Muridae rodents, with at least 423 species and seven genera in eight tribes. Members of this group, the "New World" rats and mice, display a vast array of habits and physical characteristics. Sigmodontinae range from Tierra del Fuego northward through South America, Central America, Mexico, and into the United States. They are also found on the Galapagos Islands. Two subgroups are currently distinguished: Neotomini and Sigmodontini. Several recent works question the monophyletic origin of Sigmodontinae.

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CHAPTER 17

Phylogenetic Methods for the Analysis of Parasites and Pathogens

Jamie R. Stevens

School of Biosciences, University of Exeter, Exeter EX4 4PS, UK

17.1 INTRODUCTION

A group of organisms defined by a set of shared characters are (setting aside for one moment concerns about functional convergence) generally assumed to have a common (monophyletic) origin. Cladistic interpretations, developed during the 1960s [102], sought to improve on this by using shared characters to deduce an ancestral form and assess derivatives in terms of subsequent changes in these features. Such approaches appeared less subjective and amenable to simple matrices of character presence or absence, and could readily incorporate the results of early molecular studies such as presence or absence of particular enzyme loci. Unfortunately, the results threatened to upset some of the more utilitarian classifications and often led to heated debate, for example [158]. Moreover, although cladistic methods worked relatively well for larger animals and plants with classifiable morphological traits, a lack of distinguishing characteristics (particularly morphological characters) severely hampered the application of such approaches to the study of microbial systematics including the evolutionary analyses of most parasites and pathogens. This shortcoming of the cladistic approach led to the use of total evidence-based, phenetic discipline known as numerical taxonomy [231]. The purported benefits of such an approach for the analysis of organisms with no fossil record or comparable morphology were many, of which the ability to include many characters without prior selection or designation of status (e.g., ancestral or derived) were key, and were seen to be essential to removing bias and subjectivity.

Ultimately, as our knowledge of gene function and understanding of patterns of sequence evolution and conservation has steadily increased, many of the previous obstacles to "model-based" phylogenetic analysis of parasites and pathogens have been removed. For example, many researchers work under the reasonable assumption that cellular "house-keeping" genes, which code for core metabolic pathways, tend to be conserved and biologically isolated populations will accumulate base mutations in these genes over time. So the degree of difference between homologous genes encoding proteins that serve homologous functions reflect the degree of evolutionary difference between the organisms from which such genes are sampled. Thus, genetic relationships between organisms can now be assessed and relative patterns of divergence can be quantified, and in some cases insight into dates of divergence are possible.

Of course, such generalizations have numerous exceptions; for example, large-scale analyses of multiple gene data sets sampled from evolutionary diverse genomes have consistently demonstrated that genes can be grouped into (i) informational genes, and (ii) operational genes and that the these categories show differing patterns of evolutionary relationships in the eukaryotes [205,206] with different patterns of similarities to either archaea or eubacteria dependant on the informational or operational classification of the gene investigated [205,206]. Further cases where gene evolutionary ancestry is not equivalent to species evolutionary ancestry are discussed in Box 17.1.

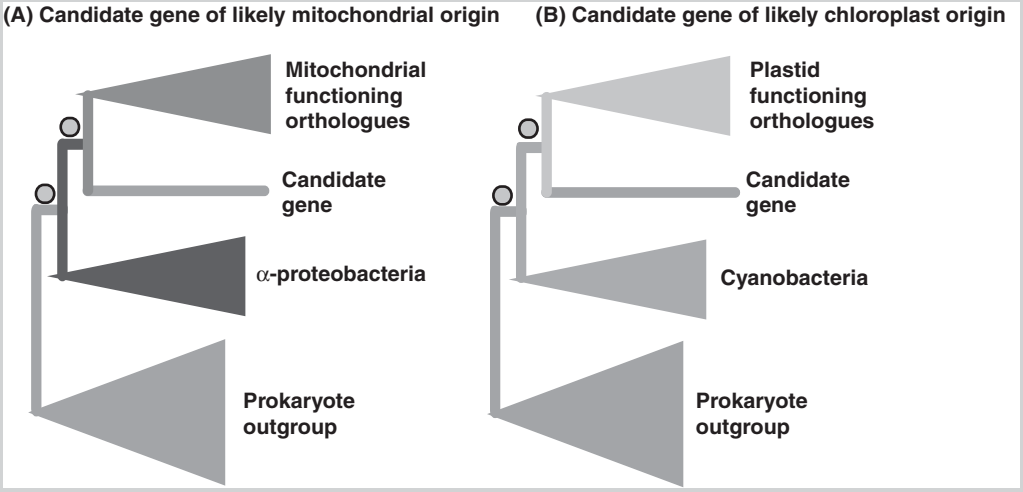
A range of powerful probabilistic and model-based methods is now available for the phylogenetic analysis of parasites

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

BOX 17.1 – DIFFERENT PATTERNS OF GENE ANCESTRIES IN PARASITES

Phylogenetic analyses have demonstrated that genes residing on parasite genomes have different evolutionary origins suggesting that many of these genomes are at least partially chimeric in nature [203]. This phenomenon is not restricted to parasites and is likely to be fundamental to both prokaryote evolution and the origin of the eukaryote cell [54,160,179,210]. Comparative genome analyses [205] have demonstrated that informational genes (functioning in translation, transcription, and replication, and incorporating GTPases, vacuolar ATPases and the majority of the tRNA synthetases) were most closely related to those of the archaea included in their study (*Methanococcus*), whereas the majority of eukaryotic operational genes (including amino acid synthesis, cofactor biosynthesis, energy metabolism, intermediate metabolism, intermediary metabolism of fatty acids, regulatory functions, nucleotide and phospholipids biosynthesis) were most closely related to those of *Escherichia coli*. Such a finding implies that the contents of eukaryote genomes may be a chimera of two distinct prokaryote lineages [205]. Follow-up analyses, with increased genome and species sampling [206], demonstrated that many individual tree topologies overlapped, suggesting that the tree of life was more akin to a ring rather then a tree and that eukaryotes represented a chimera, that is, the node that connected the tree to form a ring [206].

Many parasitic genomes are marked with comparatively recent cases of HGT. HGTs have been observed between eubacteria and pathogenic eubacteria (reviewed in [179]), and from prokaryote lineages to parasitic protozoa (reviewed in [203]). The best method for identifying cases of HGT is the use of phylogenetics to demonstrate a gene phylogeny that differs from the species phylogeny with strong support. Phylogenomic analyses of the human parasite *E. histolytica* genome identified 96 putative prokaryote to eukaryote HGT events based on a transfer being the most appropriate explanation of the tree topology observed [150]. Additional frequent cases of gene transfer have occurred from the progenitor genomes of endosymbiotic organelles to eukaryotic genomes. These include genes of plastid origin in the Apicomplexa (e.g. [269]), and genes of mitochondrial origin in the eukaryotes including the amitochondrial eukaryote parasites *Giardia intestinalis*, *T. vaginalis*, *E. histolytica*, and the Microsporidia [30,81,82,211]. The mitochondrial progenitor was an α-proteobacteria or a close relative [138,268] and the eukaryotic plastids originated from a cyanobacterium or a close relative [161,268]. Consequently, eukaryote genes that form strong sister relationships on phylogenetic trees with either α-proteobacteria and cyanobacteria potentially originate from the genome of these organellar progenitors. The schematic trees shown below demonstrate the phylogenetic relationships indicative of (A) mitochondrial origin and (B) plastid origin. Nodes marked with gray circles represent important nodes where strong topology and bootstrap support is required to infer endosymbiotic origin.



and pathogens. These will be briefly reviewed in this chapter, together with coverage of the many factors that continue to adversely affect our ability to reconstruct and interpret the phylogenies. Phylogenetic investigation can be key to understanding numerous important factors connected with parasite biology beyond simple evolutionary relationships of parasites; these include identification of parasite/pathogen diversity from DNA samples alone; host parasite coevolution; host switching; identification of genes originating from endosymbiotic gene transfers (EGT) enabling the pinpointing of ancient endosymbioses, horizontal gene transfer (HGT); identification of genetically novel pathways (differences between host and parasite) as therapeutic targets (HGT and EGT can also be important here), patterns of parasite transmission and parasite biogeography. All of these lines of investigation will be discussed in relation to the phylogenetic methods reviewed.

17.2 THE PHYLOGENETIC PROCESS

17.2.1 Source Material

The starting point for any genetic analysis is the organism from which the evolutionary marker is sequenced. But what is the organism and how was it identified? In many instances, more than two centuries of alpha-taxonomy (morphological, biochemical, and pathological characterization) are used to specify the organism from which DNA is extracted, but this process is far from infallible, and all too often we see errors in that initial *a priori* determination. Even sequence data lodged with EMBL (http://www.ebi.ac.uk/embl/) and GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) is only as reliable as the authorities providing the original determination. Alpha-taxonomy is based largely on the holotype concept, whereby a single specimen is declared as the "name-bearing" type, so that all other specimens can be compared with that holotype [124]. In microbiology and parasite/pathogen research (as in all areas of biology), it is important to be aware of the limitations of morphological characterization and of the existence of clonal lineages [264] and cryptic species [270]. The example of the oyster parasites, *Perkinsus marinus* and the alveolate heterotroph *Colpodella* demonstrates how morphological data can contrast the results of phylogenetic analyses. These two Alveolates were demonstrated to be morphologically identical bar the free-living habit of *Colpodella* (e.g. [230]) but in phylogenetic analyses branch in very different positions within the Alveolate radiation [34,201].

Nevertheless, although in metazoan biology at least the holotype system is generally well established, the deposition of holotypes of many parasites and pathogens has been at best *ad hoc*, with strains of interest kept in the freezers of many different laboratories. Fortunately, with the advent of managed collections, for example, the American Type Culture Collection, this system is becoming formalized, facilitating structured and repeatable comparison of pathogen strains and providing a resource for continuing studies. Ultimately, the development of a "holotype" genome concept[1] for all parasites and pathogens, for example [147,258], will allow improved evolutionary and epidemiological analysis of many disease agents.

17.2.2 DNA Sequencing and Alignment

With careful design and choice of primers, tissues free of symbionts, and skilled laboratory techniques most problems of DNA and/or RNA[2] extraction and contamination can now be either detected or avoided, even if not eradicated. For example, analyses of *Plasmodium* cultures have shown persistent *Mycoplasma* contaminations [218]. Problems of sample mislabeling can plague even the best-run laboratories, sometimes leading to erroneous suspicions of contamination. Indeed, many genome projects contain large numbers of genes, the presence of which can only be explained by contamination; this can be easily assessed by BLAST[3] searches of genome unassembled reads using a suitable SSU rDNA seed. Analyses of early versions of the *Cryptosporidium parvum* genome project revealed possible eubacterial contaminants (personal communication). DNA contamination can however be a product of a parasites biology, for example, intraerythrocytic *Plasmodium falciparum* can spontaneously take up and express DNA from host red blood cells that can contain residual nuclear material [49]. Consequently, the human-type ALU and LINE repeat elements, detected in *Plasmodium* sequencing projects were demonstrated to be the product of host contamination [50].

But what bits of DNA are to be sequenced? For phylogenetic comparisons, it seems overly ambitious to sequence the whole genome (although even this is changing [19] and other studies suggest that large sampling is necessary to resolve ancient evolutionary relationships [208]), so particular genes or regions are selected. Conserved or variable regions,

[1]When a species is described and named, the name given to a defined individual specimen is declared as the holotype (or name-bearing type). This holotype specimen then becomes the reference against which any other individual can be compared to see if it fulfils the same species concept and so merits the same name; in the case of the holotype genome concept, the genome becomes the holotype and all subsequently analyzed genomes are compared against the first designated genome (or set of designated genetic markers).

[2]With the growing focus on multiple gene datasets for phylogenetic analyses Expressed Sequence Tag (EST) studies are increasingly being used as a method for sampling multiple gene datasets as phylogenetic markers, for example [94].

[3]BLAST is a sequence comparison algorithm "optimized for fast coverage of sequence databases" and searches for optimal local alignments to a query sequence [2]. BLAST is a heuristic search method that searches for patches of similarity over a certain length dependant on a pre-determined similarity score. The patches of similarity can initially be as small as three characters. When a similarity threshold is hit the alignment comparison is extended in both directions over the sequence to find a high-scoring pair (HSP). HSPs that meet a certain criteria will be reported and ranked in the BLAST report. BLAST reports include percentage identity and the S score which is the sum of each of the letter-to-letter and letter-to-null position comparisons over the alignment [2]. Recent modifications of the BLAST programs report an e-score, a probabilistic evaluation given the breadth of available data in the database searched that the two sequences are similar by chance [2].

or both, may be sought, depending on the question and the perceived closeness of the organisms to be compared – thus, a judgment based on current classifications has already been made, which, when dealing with microorganism with few and often contentious morphological features, risk being seriously misleading (see Section 17.2.1 and discussion of the oyster parasite *P. marinus* above). Further limitations may be encountered due to the nature of structure, function and homologies of the sequences and the functional product chosen for analyses. Additional problems can arise from the genetic signature of ancient endosymbionts and the frequency of HGT, for example, the plastid-like genes recently reported in trypanosomatids [159], or the apicoplast derived from secondary endosymbiosis of a eukaryotic algae in Apicomplexa, for example, *P. falciparum* and *Toxoplasma gondii* [167,269] (see Box 17.1).

Gene structure and homology can have a major effect on alignments, especially when taxa are only distantly related, whereas pseudogenes and pseudogene fragments can further undermine the process of both sampling and analysis. In the worst case, lack of homology may mean seeking apparent alignments between different genes, or genes that are different representatives of a similar gene family (e.g., absence of orthologous genes, coupled with inadvertant sampling of paralogues). This can become extremely complicated when ancient and local paralogues occur by an advanced pattern of duplication coupled to mosaic gene loss, generating complicated gene families [203] (Box 17.2). Such considerations make total genome sampling of numerous lineages an attractive and safer approach, but until very recently almost impossible on a significant scale.

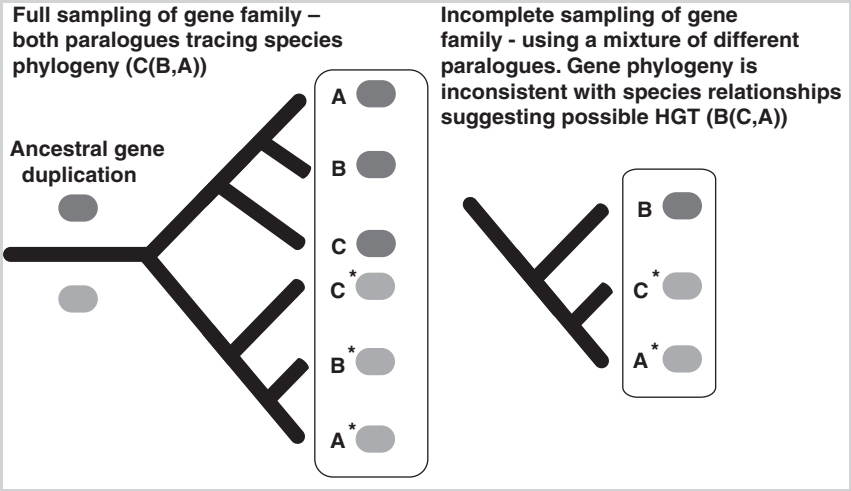
In addition, phylogenies do not form an adequate means of testing an evolutionary hypothesis without appropriate taxon sampling both within the test group and with relation to a justifiable outgroup. Without this there is no adequate alternative hypothesis available for evaluation and consequently the phylogenetic experiment is flawed. In truth, the uncharacterized extent of the diversity of life [152,154] both extant and extinct suggests that any taxon sampling for a phylogeny is, in fact, a compromise. Therefore, the process of taxon sampling is based on a balanced judgment that should be justifiable in response to the research question proposed.

Interestingly, molecular investigation combined with phylogenetic analysis has been the principle tool for investigating the diversity of microbial life in the environment (e.g. [47,152,154,201]) and can be used to investigate pathogen/parasite diversity both in tissue samples and in the environment. Such investigations have led to some interesting observations with regard to parasite diversity — see Box 17.3 [171] — but have consistently demonstrated that the molecular diversity of microbes is vast, suggesting that even comprehensive sampling of culture collections probably does not reflect the true diversity of life.

During the process of gene sequence alignment, automated alignment programs will not detect errors in original base determination. Such errors can introduce frameshifts where sequence data have been read from gels, as many until recently have been; consequently, a visual check of apparent alignments is always required [263]. Visual alignment checks are also required as many alignment programs do not deal well with highly variable sequences, this becomes even more important with the increase in quantum evolution or

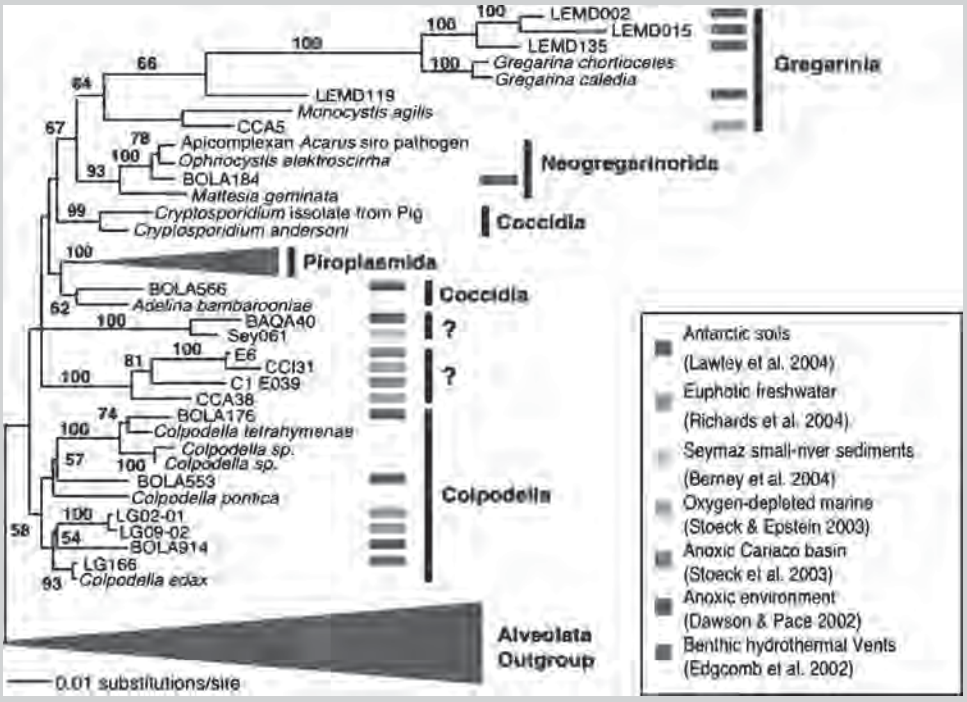
BOX 17.2 – THE POTENTIAL EFFECTS OF RANDOM PARALOGUE SAMPLING ON GENE TREES

This figure demonstrates a hypothetical scenario to show how the products of gene duplications might be confused with HGTs if sampling of genomes is incomplete or genes have been lost. After Richards et al. [203].



BOX 17.3 – ENVIRONMENTAL/TISSUE PCR AND ASSESSING PHYLOGENETIC DIVERSITY TO INVESTIGATE MICROBIAL DIVERSITY

A common approach to evolutionary analyses of microbial organisms is sequencing and phylogenetic analysis of the SSU rDNA (e.g. [234]). These genes can be sampled with relative ease from microbial cultures and, more recently, from environmental samples using selective PCR primers [83]. Such approaches can be applied to tissue samples to either identify specific parasites and pathogens, or to analyze the broad diversity of parasites present in tissues [15,278]. Heterogeneous populations of SSU rDNA amplified from environmental DNA and sampled using gene library methods have demonstrated an extraordinary molecular diversity when analyzed using molecular methods [47,152–154,201,204]. The branching position of several environmental SSU rDNA genes suggests that they are close relatives of known parasite lineages. Many of these sequences have been sampled from environments which are, from a human perspective, "extreme," such as deep sea hydro-thermal vents. These analyses suggest a large diversity of parasites or undescribed free-living sister lineages to known parasites remain currently unstudied [171]. The phylogeny below demonstrates an example of the unique molecular diversity sampled from several environments [18,47,143,204,248,249], including hydro-thermal vents [60] that cluster with known Apicomplexan parasites. This tree is a subset of a comprehensive review by Richards and Bass [202] of eukaryotic environmental SSU rDNA genes. The tree was calculated using a LogDet distance method with proportion of invariant sites accounted for. The topology is the best scoring tree from 100 heuristic searches. The topology support values are calculated from 1000 bootstrap replicated using the same method described above. See color plates.



sequence divergence incorporated within a sequence alignment. The choice of alignment program and its underlying algorithm can also affect the final alignment, and guidelines concerning the "correct" values to apply to such parameters as "gap cost" remain poorly understood, though extrinsic factors such as secondary structure are now commonly being considered as an aid to accurate alignment [105].

The analyses of gene sequences after alignment and sometimes in response to the results of phylogenetic results provide a number of important lines of investigation useful for understanding evolutionary relationships. Increasingly, shared derived genetic characters such as gene fusions [125,202,237,238] insertion/deletions within conserved open reading frames [7,12,14,202], location of introns [63]

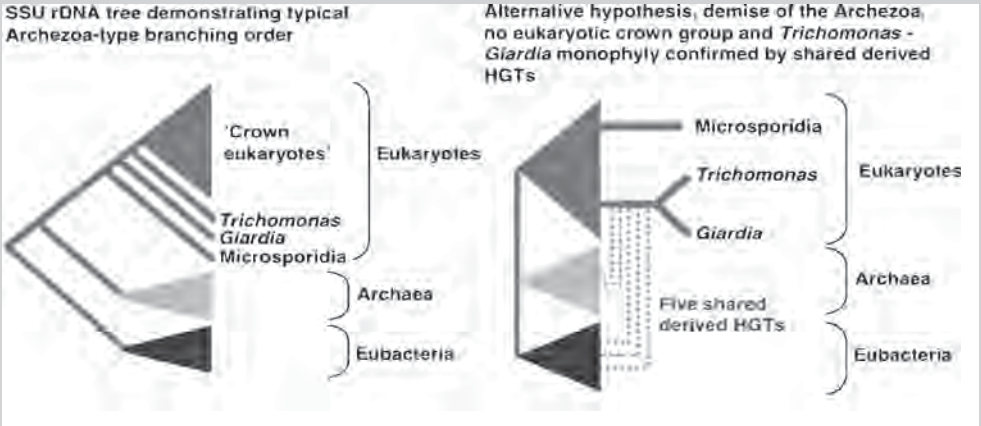
and gene order within operons [22] are emerging as useful tools for investigating evolutionary relationships. In addition, having constructed a phylogeny, shared derived features, such as HGTs [4,104,168], can also provide important characters for informing evolutionary relationships (see Box 17.4). These characters enable trait polarization, for example, rooting between groups of taxa that possess derived genetic characters. This has an advantage over sequence phylogeny that is liable to systematic artifacts (discussed in Sections 17.2.3–17.2.5, e.g. [62,246]), and cannot be used to pinpoint the root of phylogenetic trees without additional assumptions, including identification of an appropriate outgroup. Like all characters used for evolutionary inference, they can be homoplastic and in some cases can show evolutionary patterns consistent with subgenic gene transfer or convergent evolution. Investigations of gene character synapomorphies in the sexually transmitted, long branching parasite, *Trichomonas vaginalis*, and the enolase gene from the apicomplexan parasites (including *Plasmodium*) has demonstrated the fallibility of such gene characters [12,132].

BOX 17.4 – SHARED DERIVED HGT IS THE BEST EVIDENCE FOR SISTERHOOD OF *TRICHOMONAS* AND *GIARDIA*

The placement of these two parasitic, anaerobic eukaryote lineages within the eukaryotic tree is a contentious issue [113,232,233]. There are a number of gene phylogenies supporting *Giardia* and *Trichomonas* sisterhood [10,62,113,165] consistent with interpretations of morphological data [33,227,228]. However, gene sequences from these two species form very long branches on gene trees, a result of the comparatively fast rates of sequence change that these organisms undergo [62]. These results mean that their placement in phylogenies should be viewed with caution as their monophyly may potentially be an artifact produced by LBA [62,193–195,246].

The identification of multiple shared derived HGTs supports the monophyly of these two anaerobic flagellate lineages more convincingly then any standard phylogenetic investigation thus far produced. Minotto et al. [168] published the sequence of a carbamate kinase (*CBK*) gene from *T. vaginalis* and demonstrated that this gene is probably a shared derived HGT, which would have been present in the common ancestor of both *Trichomonas* and *Giardia*. Although their analyses did not correct for site-rate variation and did not confidently pinpoint a prokaryotic donor lineage, *T. vaginalis* and *Giardia intestinalis* formed a monophyletic group with 70% bootstrap support.

In most eukaryotes, the first step in glycolysis is catalyzed by hexokinase. However, *Giardia intestinalis*, *Spironucleus barkhanus* and *T. vaginalis* contain a distantly related enzyme called glucokinase, also found in eubacteria [104]. These parasite enzymes form monophyletic long branches in the glucokinase phylogeny consistent with a single common origin and cluster weakly with cyanobacterial sequences. The second enzyme in glycolysis is glucosephosphate isomerase (*GPI*). The *GPI* gene from these three parasites is monophyletic, and groups within the eubacteria separate from the gene ortholog family found in other eukaryotes [104]. Recent analyses of the prolyl-tRNA and alanyl-tRNA synthetase genes demonstrated likely HGT events from within the archaea radiation to the shared ancestor of *Giardia* and *Trichomonas*. In the alanyl-tRNA synthetase analyses, the donor lineage could be pinpointed to the Nanoarchaeota with robust phylogenetic support [4].

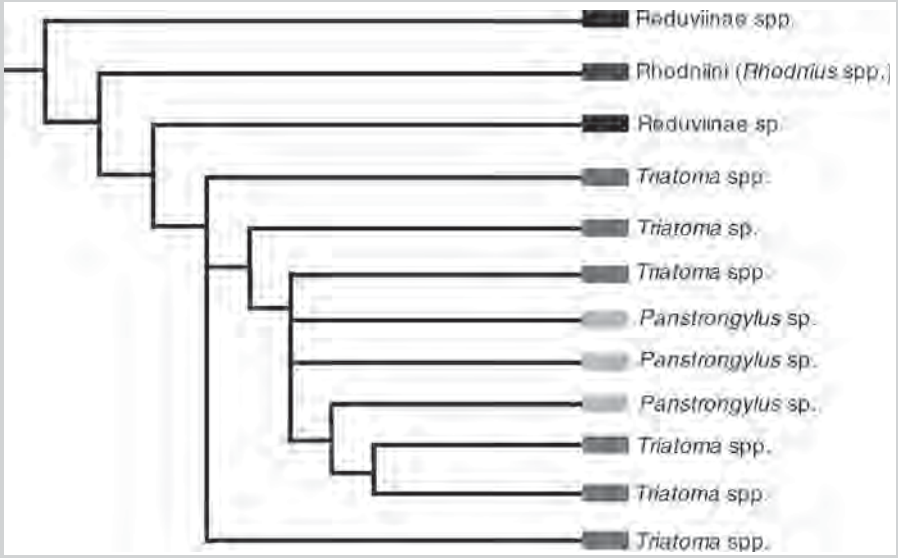


But what of the function of the chosen sequence? If the organisms being compared are truly monophyletic then sequence function may not be so important. But with polyphyletic or paraphyletic groups, comparisons based on conserved sequences will be poorly informative of relationships between extant organisms. For example, the Triatominae – the vectors of *Trypanosoma cruzi* – are defined on the basis of their blood-sucking habits, but are believed to derive from predatory Reduviidae. If they are monophyletic (i.e., all derived from a single blood-sucking ancestral form), then their phylogenetic relationships may well be deduced from differences in "house-keeping" genes such as cytochrome oxidase [80]. But if they are polyphyletic (i.e., derived from a series of different reduviid ancestors), as biological evidence suggests [192,222], then an analysis based on such genes can inform only about these different ancestral forms – not about the Triatominae themselves. In such a case, a comparison of genes involved directly with the characteristic that defines them – in this case haematophagy – would be required; see Box 17.5. However, recent analyses demonstrate that different "tribes" of blood-sucking Reduviidae are paraphyletic, independent of the origin of the blood-sucking phenotype.

So, logically, we might seek to include data analysis from several different gene sequences, derived from a range of (ideally independent) metabolic pathways and functions within an organism. But how many? There is no doubt that small data sets can lead to unconvincing results, for example [123], and differences due to introgression may not be detected by limited sequence comparisons [245,261]. But how can we tell that the overall data set is large enough? When phylogenetic trees agree with previously held concepts? Or when they no longer change significantly with the addition of further data? [48]. We assume here that the "previously held concept" is rational, based on a different data set, such as morphology or other biological characters. Adding genetic data may not be additionally informative – for example, adding nuclear DNA sequences to an analysis based on mitochondrial DNA sequences may not give significant changes to phylogenetic topologies [170], reflecting the fact that two data sets are following similar evolutionary routes; alternatively, the combination of such data (e.g., nuclear and mitchondrial gene sequences) may lead to insight into major evolutionary switches [16,17]. In a recent phylogenetic study of eight yeast genomes, Rokas et al. [212] obtained a fully resolved species tree using 106 widely distributed orthologous genes. Significantly, in associated incongruence analyses they demonstrated that a *minimum* of 20 genes were required to obtain comparable results – substantially more genes than commonly used in phylogenetic studies, but only a small fraction of any genome. The *minimum* prediction of Rokas et al. [212] may only be a relevant to analyses of organisms as closely related as the ascomycete yeasts. Rodriguez-Ezpeleta et al. [208] used a phylogenomic approach to test the monophyly of the primary plastid

BOX 17.5 – TRIATOMINAE AND PARAPHYLY OF THE BLOOD-SUCKING HABIT

Schematic Reduviidae (Assassin bugs) phylogeny, showing paraphyly of the blood-sucking Triatominae (Rhodniini [blue], *Triatoma* [red], *Panstrongylus* [yellow]), relative to predatory Reduviinae "outgroup" species [black]. Summarized from the phylogenetic analyses of Paula et al. [192] based on mitochondrial 16S ribosmal RNA gene sequences. Note that the *Triatoma* and *Panstrongylus* branches are paraphyletic and do not form an exclusive clads. See color plates.



lineages (plants, red algae and glaucophytes). This represents a much more ancient phylogenetic investigation than the analyses of Rokas et al. [212] and consequently Rodriguez-Ezpeleta et al. [208] concluded more than 100 genes were required to recover monophyly of the primary plastid lineages. In light of the many factors already discussed, such an approach is very difficult for broad analyses of ancient relationships and only a very limited number of studies have so far followed this approach, using four [10], six [97] and 22 [8] genes. Moreover, as Taylor [259] alludes to and Rodriguez-Ezpeleta et al. [208] demonstrate, more genes are probably needed the deeper we go and as morphological comparisons become ever less meaningful.

Practically, sequence alignment and the associated problem of identifying true homology between variable sites and portions of sequences remains one of the most problematic areas in molecular phylogenetic analysis. Alignment can be performed by one or a combination of three main approaches: (1) using one of a range of specialist alignment programs with various weighting options and gap penalties, for example, CLUSTAL-X/CLUSTAL-W [262] or T-COFFEE [177]; (2) on the basis of secondary structural and functional domains, for example secondary structure in ribosomal sequences [174]; and (3) by eye, often in relation to previously aligned sequences. Increasing the number of taxa may be accompanied by problems of hypervariability at some sites and saturation of changes at others, resulting in reduction of informative sites suitable for inclusion in phylogenetic analyses. Sites which are informative between closely related taxa may introduce "noise" at higher phylogenetic levels, resulting in a loss of definition and reduced bootstrap support (see below); such sites may be excluded from broad analyses, provided sufficient data remains to be able to perform a meaningful analysis. This leads to a process of masking sequence alignments and the removal of heavily gapped regions, hypervariable regions, or sequence regions that cannot be aligned with confidence.

17.2.3 Phylogenetic Methods

Much has been written about how the choice of phylogenetic method can affect evolutionary reconstruction [84,115,137,176,190,220,236]. However, it is worthwhile considering that phenetic and cladistic methods, such as the parsimony approach, were originally developed for the analysis of morphological and morphometric data – not for analyzing DNA or amino acid sequences – and considerable debate has centered on the suitability of phenetic and cladistic methods for analyzing sequence data [40,66,116,253].

Nevertheless, as the use of such methods to analyze DNA data has increased, various weighting schemes and constraints have been introduced, for example Dollo parsimony [64], to reflect better the complexity of the evolutionary processes being modeled. Such schemes, however, do not afford the flexibility to incorporate the full range of parameters that explicitly model-based methods can offer – see below.

There are four main categories of phylogenetic methods in widespread use – distance, parsimony, Bayesian, and maximum likelihood (ML) analysis – the relative merits of which have now been explored directly by a range of simulation studies [40,121,175,272]. Although, for example, parsimony and ML methods require greater computing power than, for example, distance methods, this is unlikely to be so limiting in the future.[4]

17.2.3.1 Models of DNA evolution Beginning from relatively simple models, such as the Kimura two-parameter model [134] and Felsenstein's F81 model [69], models of DNA evolution have been developed through increasing levels of complexity – for full details see [66,84,176,190]. Later, more complex models, include likelihood methods based on models of sequence evolution in which all character state changes and sequence parameters are definable. The rationale for such an approach is that knowledge of how sequences evolve can enable more accurate prediction of the probability (= frequency) with which mutation events will occur. But choice of an appropriate model is paramount to accurate evolutionary reconstruction. Good fit to an accurate model should provide a robust analysis. Moreover, use of an inappropriate model can be seriously misleading. Fortunately, modern analysis packages, for example PAUP [254] and TREE-PUZZLE [221], allow parameters to be estimated from the data, such that a model can be selected by an essentially iterative process of improving log-likelihood scores. The recent development of an add on to PAUP – MODELTEST [197] – provides a relatively quick method for assessing 56 possible pre-set models for DNA sequence phylogeny, successively varying different parameters; tests for improvements in the accuracy of successive models are performed automatically using likelihood ratio tests. Although quick, however, MODELTEST in some cases may suggest an inappropriate model, as the step-wise likelihood ratio tests select the model (and associated parameters) from the point in the process that MODELTEST fails to return an improved result. For example, if model 6 is not a significant improvement on model 5, then MODELTEST selects model 5 and then does not consider comparisons made to models 7–56 (although the likelihood score of all models is accessible elsewhere in the MODELTEST output); it also does not test all possible combinations of corrections for site rate variation. A robust alternative to MODELTEST is MODELGENERATOR [129], which can also be used to find the best model and substitution matrix for an amino acid alignment.

17.2.3.2 Distance methods Distance methods include some of the earliest approaches used for taxonomic and evolutionary studies and despite significant advances in other

[4]In a single chapter, in a book of this scope, it is not possible to cover all the methodological details of the many phylogenetic methods currently available and readers wishing to extend their understanding of any particular method are urged to refer to one of the many excellent texts now dedicated to this subject [70,84,176,187]; see also Griffiths and Stevens [89] for a review of Web-based resources.

fields, that is, cladistics and likelihood methods, they remain in widespread use today, primarily because of their speed and relatively low computational resource requirements. The limitations of many simple distance methods, for example unweighted pair-group method using arithmetic averages (UPGMA), are now well recognized. However, increasingly sophisticated distance measures have been developed.

Significantly, tree construction by pairwise distance measures proceeds in two main steps: (1) for all pairs of sequences, a genetic distance is calculated based on sequence dissimilarity weighted against a model of sequence evolution, the results reflect the average number of changes per site since each pair of sequences diverged; and (2) the resulting matrix of distances is then used to construct a phylogenetic tree by one of the many available least squares clustering methods, for example UPGMA, neighbor-joining [219] and Fitch–Margoliash methods [73], which attempt to fit the distances to a tree. Ultimately, accurate estimation of the genetic distance is more important than the choice of clustering/tree building method; as shown previously, use of an appropriate model of evolution is essential for accurate tree reconstruction [239].

However, the conversion of taxon/character data into pairwise distance measures inevitably involves a reduction in information content compared with the original sequence data, with information reduced to one average value per sequence pair. Nevertheless, the two-step nature of their calculation allows genetic distances to be calculated by a wide range of measures, including those based on robust substitution models (of which many are now available, see Section 17.2.3.1), to take account of such factors as multiple mutations. Reduction in computational complexity also permits the analysis of larger data sets than possible with parsimony or ML methods; moreover, certain distance methods, for example neighbor-joining, have been shown by simulation studies to perform well against such complex methods [272] under certain specific circumstances, namely when mutation rates are approximately equal (or at least, are not significantly unequal) across different lineages [149,235]. Some clustering methods, however, for example UPGMA, are very susceptible to differences in mutation rates between lineages [235] and sequence composition bias [76], which may affect both DNA-based and protein-based phylogenetic reconstructions.

Consequently, "data selective" distance matrix methods, for example LogDet (which is particularly important when analyzing some parasite genes, for example, the *P. falciparum* genome which is >80% AT rich [79]), have been developed [219,239]. LogDet distances (also known as paralinear distances) are particularly adept, in some cases, at reconstructing relationships among sequences that have evolved by a non-homogeneous process (in relation to base composition) [139,149]. Another favored sophisticated matrix is the General Time Reversible (GTR) matrix that assigns individual probabilities to each type of change based on observations made from the data [90]. Many distanced methods can include a correction for site rate heterogeneity, such as a Gamma distribution and/or correction for the proportion of invariant sites. These parameters are estimated in comparison to a given phylogeny. Clearly the accuracy of the parameters estimated depends on the use of a reasonable tree topology invoking a chicken and egg scenario – which comes first? However, this problem can be overcome with iterative tree topology calculations and parameter adjustments. Indeed, Yang [279] suggests that the topology used for parameter calculation only has to be reasonable to achieve good estimates for model parameters.

Thus, with the advent of accurate, robust model-based genetic distances, coupled with the other longstanding benefits of distance matrix-based approaches, it appears that distance measures will continue in use as rapid, relatively accurate options for phylogenetic analysis in the near future.

17.2.3.3 Parsimony methods

17.2.3.3.1 Background Cladistic methods [103] focus on finding the shortest, most parsimonious phylogenetic tree(s) (MPT) to fit the data, that is, the tree requiring the smallest number of steps or invoking the least amount of sequence changes. Parsimony can be used to analyze a range of data types, which can be morphological, physiological, biochemical, molecular and even linguistic – the only requirement being that the data can be translated (coded) into discrete characters. For molecular data, the changes (steps) between taxa are the individual nucleotide or amino acid substitutions. The advantages of parsimony compared with distance methods are (1) that all informative characters are considered, rather than summarized by conversion to a pairwise distance; (2) all possible solutions (MPTs) can be considered and if required combined into a consensus tree; and (3) a range of related search strategies allow even very large data sets to be analyzed. However, as noted, parsimony works only on variant characters and thus alignment information common to all taxa analyzed is excluded; the basic assumption of parsimony – that evolution proceeds economically – is also open to theoretical question. Moreover, this method is not suitable for all situations [116], particularly where rapid and/or dissimilar change occurs within a data set.

Cladistics relies on several key methodological factors. Firstly, cladistics aims to identify sister-group hierarchies, such that two taxa are more closely related to each other than they are to all other taxa. Taxa are grouped on the basis of shared, discrete similarities in the data, allowing hierarchical branching trees (cladograms) to be constructed which represent the relationships between the taxa being analyzed. As outlined above, data are coded as characters, with two or more discrete character states, for example, in DNA sequence data each position within a sequence alignment will equate to a character, whereas character states will be one of four bases: A, C, G, T (in some instances, users may elect to code gaps as a fifth character state). Closely related taxa will thereby exhibit a high degree of similarity between the character states of each of their characters. Secondly, cladistics relies on the ability to

recognize a sister-group (or outgroup) to provide polarity (evolutionary direction) to any changes occurring in characters between taxa within the ingroup (usually the group of interest).

Having established evolutionary polarity, characters states may be recognized as ancestral (pleisiomorphic) or derived (apomorphic). Shared derived changes (apomorphies) are referred to as synapomorphies and ultimately are the basis of all robust evolutionary trees (see [136] for full details).

17.2.3.3.2 Weighting schemes Since they were first used to analyze molecular data [59], attempts to improve the accuracy of parsimony methods by better modeling the underlying processes of molecular evolution have led to a gradual but almost continuous increase in their complexity. Consequently, many different versions of parsimony methods are now available for analyzing molecular data, which can be divided into unweighted and weighted methods. As their name suggests, unweighted methods do not account for the differences in probability of the different substitution events, sequence content bias, and so on, which can occur in molecular sequence data, although, because virtually all mathematical models are crude approximations of reality, weighted methods do not always lead to improved performance [74]. Weighted parsimony methods take two forms: *a priori* and *a posteriori*. The latter form is common to parsimony analysis of all forms of data and is hypothesis/tree dependent, and includes approaches that weight successive analyses according to levels of homoplasy within the tree. Alternatively, *a priori* weighting attempt to account for the different probabilities of molecular evolutionary events and factors such as frequency of transitions (generally common) and transversions (generally less common); base composition bias; secondary structure in rRNAs; codon position in protein gene sequences. Ultimately, the complexity of such schemes may approach that of true model-based, for example, likelihood, methods; see [70] for a full discussion of the statistical properties of parsimony methods.

17.2.3.3.3 Tree searching methods In most if not all cases, the relationships between taxa in a given data file may be explained by many different arrangements of the characters states within the data, and for each arrangement relationships between the taxa may be represented as a phylogenetic tree of a length equal to the number of steps invoked. As stated, the primary objective of parsimony methods is to find the tree (or trees) which explain(s) the changes within the data by the smallest number of steps, that is, the most parsimonious solution. As the number of taxa in an analysis increases, however, so the number of all possible arrangements of these taxa also rises, until for 10 taxa the number of possible arrangements is greater than 2,000,000, whereas for 15 taxa the number of possible trees is almost 8,000,000,000,000. [136] Thus, in the analysis of all but trivial data sets, consideration of all possible trees in order to find the shortest tree(s) is simply unrealistic and, in consequence, a number of methods for searching through the many nonoptimal trees have been developed. Typically, these methods trade accuracy for speed in their search algorithms. For 11 or less taxa, it may be possible to perform an exhaustive search; for larger data sets, however, the use of approximate or heuristic search methods is essential – see, for example [136,256] for further details.

In an ideal situation, a given data file would contain sufficient synapomorphies (shared derived characters) and no conflicting character state changes (in sequence analysis this is equivalent to no reversals or saturated nucleotides), such that when analyzed, the data file would produce one single MPT to fit the data. However, given the often large number of saturated sites found at some aligned positions, for example, third positions of protein coding gene sequences, this is rarely the case and not infrequently two or more (sometimes many more) MPTs (different trees of equally short length) are found that fit the data. In such case, the user may opt to consider either or all of the following:

- Each of the MPTs.

Subtrees, that is, those parts of each of the MPTs which are common to all the MPTs.

- A consensus tree, that is, an "average" tree constituted from all of the MPTs.

Fortunately, programs such as PAUP [254] and PHYLIP [71] that are commonly available for phylogenetic analysis, provide users with simple ways in which to investigate these options.

17.2.3.3.4 Problems with parsimony Parsimony methods generally perform best when rates of evolution are slow, though they can also perform well when rates of evolution are high, provided that long branches on the true underlying tree are suitably broken up by appropriate intermediate taxa, thereby reducing the phenomenon known as long-branch attraction (LBA) (see Section 17.2.5.1). As first described by Felsenstein [67] and later illustrated in a simulation study by Hillis [106], parsimony will, under such circumstances, converge on the wrong tree. Unfortunately, in such a situation, measures designed to provide confidence in the resulting phylogeny, for example, bootstrap values (see Section 17.2.5.1), only serve to reinforce support for the wrong tree; moreover, of the four commonly used categories of phylogenetic reconstruction methods, parsimony appears to be the method most badly affected by this phenomenon.

17.2.3.4 Likelihood methods ML is arguably the most powerful approach to phylogenetic analysis currently available. Supported by solid statistical principles [69], such methods calculate the probability of a given tree yielding the observed data. ML methods consider all characters within the alignment as independent, and the log-likelihood of each character type at each alignment position is calculated under

a given tree topology by using a particular probability model. The log-likelihood is added for all sites. The tree topology search method is then repeated for all possible topologies (when computationally feasible) – the topology that shows the highest likelihood is chosen as the final tree. The probability model of character change is often calculated under a similar process to the distance GTR probability matrix, where individual probabilities are assigned to each type of change based on observations made from the data. Again, ML trees are often calculated using a correction for site rate heterogeneity (usually using a Gamma distribution and/or correction for invariant sites) and are estimated in comparison to a given tree topology [279]. As discussed, parameter values are estimated according to the use of a reasonable tree topology, again requiring an iterative process for model and tree optimization and meaning, ultimately, that the quality of the results is dependant on the quality of the model used. This process can be computationally intensive, consequently constraints on computation time quickly render analysis impractical for DNA and especially amino acid alignments that include many sequences. The possibility of assessing bootstrap support (see below) for a given data set is similarly limited by computational requirements. ML phylogenies can be calculated for DNA alignments using PAUP [254] and for amino acid alignments using TREE-PUZZLE 5.1 [221].

17.2.3.5 Bayesian analysis Bayesian inference is a method of statistical inference based on the Bayes theorem, proposed by the Rev. Thomas Bayes, a Presbyterian minister who lived from 1702 to 1761. His key paper out lining the Bayes theorem was published posthumously in 1763. The Bayes theorem relates to conditional probability and works by evaluating an event (a cause) conditional on another event (a consequence). Therefore, Bayesian inference is concerned with the consequences of modifying our previous "beliefs" as the result of receiving some data. The process involves taking an expression of a belief or a hypothesis about an unknown quantity/phenomenon to generate *prior probabilities* or a *prior distribution*. Some data is collected and the information is summarized as a likelihood. Bayes theorem is now used to calculate the posterior distribution or "posterior probabilities" using the formulation that "posterior is proportional to the prior times likelihood." A posterior distribution can then be used as an amended prior for further experiments. Consequently, Bayesian probability is a way of measuring the degree of belief there is in a proposition.

Bayesian inferences of phylogeny [120,121,164,166, 200,215] seek a set of phylogenies that maximizes the likelihood given the data. The prior probability of a phylogeny represents the probability of a tree before any observations have begun. In most Bayesian inferences of phylogenies, all trees are considered equally possible prior to any observations. The likelihood is proportional to the probability of the observations (the sequence alignment) conditional on the phylogeny [121]. The probability is evaluated based on specific assumptions about the processes that generated the observations (i.e., assumptions about the nature of mutational change in the sequence alignment) [121]. The posterior probability of a phylogeny is then the probability of a phylogeny dependant on the observations and is calculated by combining the prior and likelihood for each tree using Bayes formula [121].

Like most other methods for phylogenetic analyses, it is impossible to consider all topologies with combinations of branch lengths and models of substitution. Consequently, the Markov chain Monte Carlo (MCMC) method is used to approximate the posterior probability [121] of a tree. The MCMC process begins with either a user-defined tree, but usually a random tree and then follows a three-step process: (1) a state is randomly proposed/altered (e.g., a tree topology); (2) the probability for the new state is evaluated; (3) a state is altered dependant on whether the new state is an improvement, the new state is only accepted if it represents an improvement and rejected if not. Usually, this process is repeated many hundreds of thousands of times and sampled at given intervals [120,215]. MRBAYES [120,215] (a free and accessible program for Bayesian inference of phylogenies) incorporates a specific version of the MCMC sampling mechanism called the Metropolis-Coupled MCMC (MCMCMC) that runs several chains of MCMC search in parallel; these additional chains (usually four in total) are "heated" to differing degrees. The greater the "heat" the higher the variation in the parameters and model randomly generated for comparison. When a "heated" chain demonstrates a better probability for a state, the alternative state is accepted. This enables the MCMCMC to explore a wider area of tree space,[5] therefore limiting the chances that the tree searches will become stuck on local optima.[6] As the chains progress, the process closes in on a set of trees for which the likelihoods are so similar that accepting or rejecting an alternative state is random. At this point, the chain has converged on a stable likelihood value. It is the tree topologies recorded during this plateau that are sampled to approximate a final tree topology and a posterior probability of each topological relationship observed.

MRBAYES3 [215] includes several analytical features useful for sophisticated analyses of gene/species phylogeny. These include user-defined data and/or alignment partitions that specify alternative models to be investigated for different

[5]Tree space is a conceptual landscape (often presented graphically) representing all possible trees and their adjacencies or perturbations between trees. Every individual point in tree space (different topology) is evaluated so that some areas are high scoring. Consequently, tree space resembles a mountain range, with multiple high-scoring peaks.

[6]The concept of local optima is best explained by the following analogy: non-exhaustive searches of tree space may become stuck on local high-scoring regions of tree space, so that in an attempt to improve the tree topologies detected, the search continues to climb the local region. However, the local region may not contain the overall highest scoring region of tree space and so the search will never find the true optimum. Metaphorically speaking, the search may become stuck climbing to the top of a foothill and fail to even attempt to climb the mountain.

sections of an alignment. Different sections of an alignment can include different genes within a concatenated gene alignment, alignments with several different types of data, that is, a combination of DNA, amino acid and morphological characters. This additional feature enables accommodation of the evolutionary scenario where data subsets are likely to evolve under the same evolutionary tree, but in different ways. This is a likely scenario, as different genes have very different models of sequence evolution due to patterns of functional selection [120,215].

17.2.4 Methods of Assessing the Robustness of Phylogenetic Relationships

17.2.4.1 Bootstrap support The "correctness" of a phylogenetic tree cannot be reliably interpreted without statistical support for the evolutionary relationships presented. Bootstrap analysis [68] involves resampling the data "with replacement" to generate multiple alignment data sets. A phylogeny is calculated for each replicate data set. The number of times (often expressed as a percentage) that particular groupings of taxa (clades beneath nodes) occur within these trees reflects the support for a given topological relationship. Debate surrounding the nonlinear nature of bootstrap support is still considerable, although clarification of what such support means and how it can be interpreted continues to be improved [107].

17.2.4.2 Decay indices Bremer support [20,21] is used with parsimony analysis to assess the relative strength of clades identified within a phylogeny. Bremer support for a clade is defined as the number of extra steps needed to construct a tree (consistent with the characters) in which that clade is no longer present. Some authors make the case for the use of this method/index as a more precise alternative to the use of bootstrap support [136].

A derivative of Bremer support – partitioned Bremer support (PBS) [9] – has been developed for use with combined data sets as a tool to measure the support from each data partition for each node of a tree from a combined data matrix. The PBS value reflects the amount of support that a given data partition contributes to a given node. In addition, PBS can be used to provide insight into combinability of data, for example, morphological and molecular, nuclear and mitochondrial, and to assess the stability of nodes to the addition of new data.

17.2.4.3 Jack-knife analysis Jack-knife analysis is similar to the process of bootstrap analyses but involves resampling without replacement. Multiple copies of the alignment are generated and each replicate is subject to random character exclusion to a user-defined given proportion. A phylogeny is then calculated for each replicate and a consensus tree with node support values is produced from the replicates. This approach enables a researcher to observe the amount of signal present in an alignment that supports a particular topological relationship. A similar approach can be applied to concatenated gene alignments whereby subsets of genes are removed allowing differences in topology and topology support values to be explored.

17.2.5 Additional Considerations

17.2.5.1 LBA and outgroup selection The choice of an outgroup and the associated placement of the tree's root sets the ingroup in evolutionary context. The outgroup may comprise closely related taxa that can be presumed from prior biological knowledge to form a sister-group or, for the sake of hypothesis testing, be assumed to be ancestral.

Thus, for example, in studies of trypanosomatids free-living bodonid species have proved suitable outgroups for rooting trypanosomatid trees using a range of ribosomal and protein coding genes [3,72,162,244,271,277], whereas, in turn, the phylogenetic position of *Bodo caudatus* has been independently verified by comparison with even more distantly related species, for example, *Euglena gracilis* [93,162]; see Box 17.6 for further details.

The phenomenon of LBA is also fundamentally linked to the issue of outgroups, and inclusion of a suitable outgroup (or groups) is essential for the reconstruction of robust, meaningfully orientated phylogenetic trees. As discussed in Section 17.2.5.2, the question of trypanosome monophyly was much in debate until the late 1990s, due primarily to insufficient taxon coverage [155]. However, as several studies have highlighted, poor choice of outgroup almost certainly contributed to this issue; Hughes and Piontkivska's re-analysis of SSU rRNA data using euglenid outgroups and their contention that trypanosomes (and trypanosomatids) are paraphyletic is a case in point [122]. By using only two closely related euglenids as outgroups (excluding, for example, diplonemids), their analyses "exaggerated" the long branch between kinetoplastids and the outgroup, presumably favoring LBA between the outgroup and rapidly evolving ingroup taxa. Thus, *Trypanosoma vivax* (previously identified as rapidly evolving) branched incorrectly outside of the Salivarian[7] trypanosomes (= the mainly mammal infective, tsetse-transmitted African parasites) only when euglenid outgroups were used.

SSU rDNA have a large and diverse taxonomic sampling [47,154,181,266,267] and are relatively easy to sample from both microbial cultures and environmental samples (Box 17.3). SSU rDNA analyses can be useful for positioning

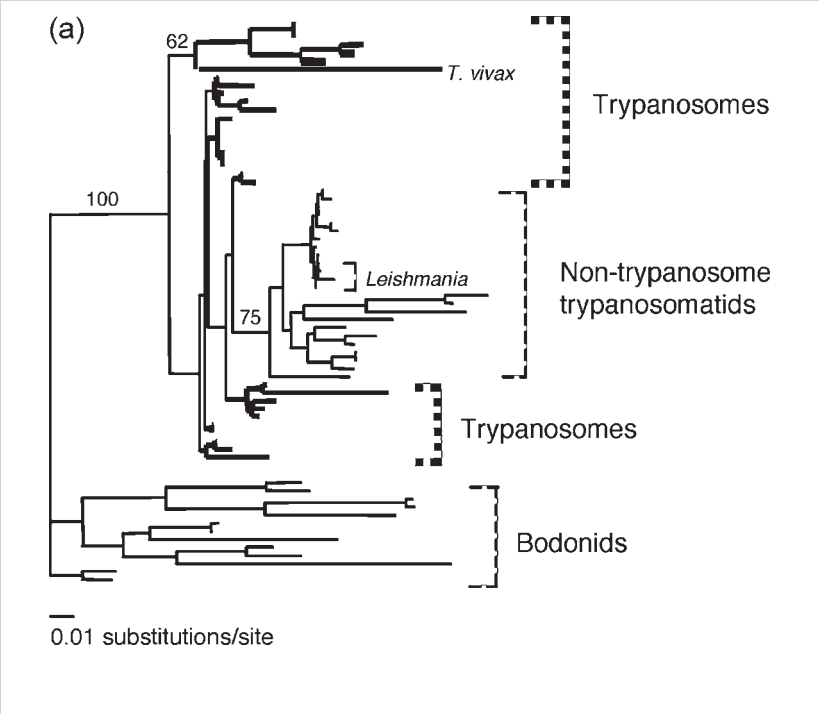
[7]The term "salivarian" is used to define a group of trypanosomes which are transmitted via the anterior end of their vector, namely by the bite of the tsetse fly in Africa. Characteristically, the group infect mainly mammals, exhibit antigenic variation and undergo cyclical development within their tsetse fly vector. The group includes the species ***T. brucei,*** which contains the two subspecies, ***T. b. gambiense*** and ***T. b. rhodesiense,*** responsible for African human sleeping sickness. At least two salivarian species, ***T. evansi*** and ***T. vivax*** have become adapted to mechanical transmission and have spread beyond Sub-Saharan Africa to infect animals in South America and Asia.

BOX 17.6 – SUITABLE OUTGROUPS FOR ROOTING TRYPANOSOMATIDS

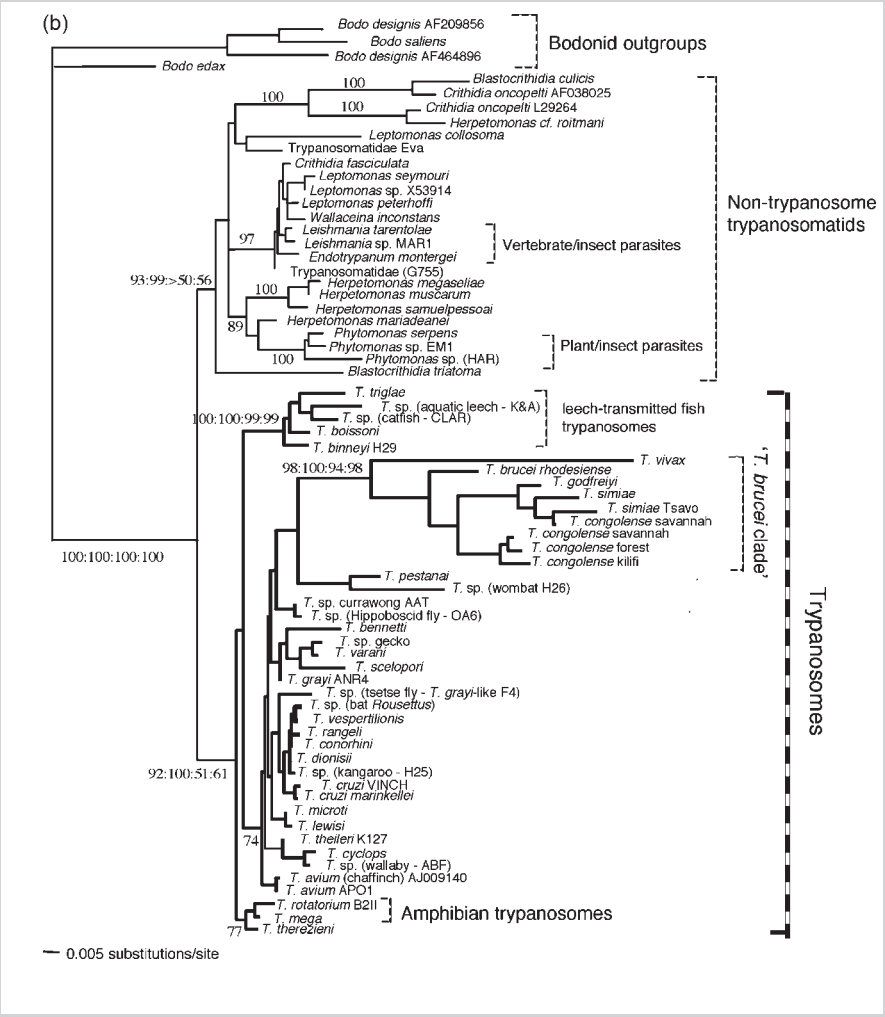
The use of distant outgroups increases the risk of LBA in phylogenies, which can result in artifactual rooting within long ingroup branches [193,256]. To investigate this phenomenon in trypanosome phylogenies, Hamilton et al. [93] constructed trees from the same alignment and successively excluded different categories of outgroup and ingroup taxa. Subsequently, they tested SSU rDNA and glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) data sets with a range of phylogenetic methods and demonstrated that with SSU rDNA-based trees topology is particularly affected by both outgroup choice and the method of analysis; indeed, exclusion of the euglenid outgroups resulted in trypanosomes appearing as a paraphyletic group (Fig. 17.6a).

Figure 17.6 (a) Phylogram constructed by ML analysis with 100 bootstrap replicates of trypanosomatid SSU rDNA sequences, excluding euglenid sequences. After Hamilton et al. [93]. (b) Phylogram constructed by ML analysis with 250 bootstrap replicates of trypanosomatid SSU rRNA gene sequences, including a broad range of outgroup taxa. Single values at nodes are ML bootstrap values. Multiple values at nodes are in order: ML bootstrap value, Bayesian support value, MP bootstrap value and ML distance bootstrap value. After Hamilton et al. [93].

However, although the limitations of SSU rDNA data are now well recognized, Hamilton et al.'s phylogenetic analyses of *gGAPDH* data provided comprehensive support for monophyly of trypanosomes, with high statistical support (86–100% depending on methodology). All trees based on *gGAPDH* sequences supported monophyly of trypanosomes and positioned them as a deep-branching lineage within the Trypanosomatidae, which was also shown to be monophyletic. This finding was robust regardless of the method of tree construction (ML, Bayesian, MP, and distance methods), or whether the alignment used comprised nucleotide or amino acid sequences. Trees were rooted outside the kinetoplastids, using the *Euglena gracilis GapC* gene, which, according to Hughes and Piontkivska [122], is considered an appropriate taxon for testing for monophyly of trypanosomes. This gene should be a reliable root, because its ratio of base frequencies does not differ significantly from that of other *gGAPDH* genes in the alignment and positions (1 and 2) are unlikely to be saturated. When considered together with published



HSP90 trees [229], a strong case for monophyly of trypanosomes is made that is independent of the SSU rRNA data. In both studies, monophyly receives robust statistical support and the outgroups are undisputed; a sufficiently wide selections of the two groups of kinetoplastids – trypanosomatids and bodonids – are included in the *gGAPDH* and *HSP90* trees, respectively, as to ensure the robustness of these results; see Simpson et al. [283] for a recent review of this topic.



lineages within established clusters on the SSU rDNA tree. DNA-based phylogenies are calculated from variance of four characters and consequently at the level of the eukaryote phylogeny SSU rDNA or indeed any DNA data set is particularly vulnerable to homoplasy, effects of compositional biases (which can also effect phylogenies of protein sequences as well [76]), and heterotachy or hidden covariation [88]. Moreover, changes in gene sequences over evolutionary time are heterogeneous, meaning that invariant sites or sites saturated by mutation can outweigh the availability of informative sites dependant on the evolutionary relationships analyzed. Inappropriate correction for differences in rates of change across a sequence alignment can produce tree artifacts. LBA is one type of artifact. LBA occurs when subsets of sequences possess radically different sequences and so form relatively long branches. In these cases, it appears that the branching position can be incorrect because of both exclusion from other branching positions based on sequence novelty, and high frequency of independent parallel changes acquired as a product of high rates of change [62,195,247].

Such problems are compounded by the inclusion of highly divergent outgroups for the purpose of rooting [194], as is conventionally used for the many eukaryote SSU rDNA phylogenies that are often rooted on the archaea (e.g. [248,249]).

One group of eukaryotes that persistently grouped at the base of the eukaryotic tree and were suggested to never have possessed the mitochondrial endosymbiont, were the mainly parasitic protozoa grouped together in the subkingdom Archezoa [31,32,232] which were defined as primatively amitochondrial. This group included the archaeamoeba (e.g., *Entamoeba*), diplomonads (e.g., *Giardia*), Microsporidia (e.g., *Trachipleistophora*, *Vairimorpha*), and parabasalids (e.g., *Trichomonas*). A species from all of these taxa have now been shown to possess genes that on phylogenetic trees cluster with mitochondrial orthologous [30,39,81,82,109,113,114,209, 211]. In *Entamoeba*, *Trichomonas*, *Trachipleistophora*, and *Giardia* proteins of α-proteobacterial ancestry localize to double-membrane bounded organelles, in the form of hydrogenosomes in *Trichomonas* and termed mitosomes in Microsporidia, *Giardia*, and *Entamoeba* [30,58,265,274]. The most parsimonious interpretation of this data is the rejection the Archezoa hypothesis for the taxa for which it was founded (Metamonada, Archaeamoeba, and Microsporidia). However, it has been suggested that the genes on archezoan genomes could have originated through HGT from other eukaryotes, or from prokaryotes other than the mitochondrial endosymbiont, thus resurrecting the Archezoa hypothesis [5,53,232,233]. Possible support for a HGT of a mitochondrial marker gene was suggested for the mitochondrial *CPN60* gene [268]. The original publication of the *CPN60* phylogeny with the diplomonad *Giardia intestinalis* sequence shows a long-branching *Giardia intestinalis* and *Entamoeba histolytica* monophletic relationship [211]. This relationship suggests an HGT event between *Giardia intestinalis* (a flagellate parasite) and *E. histolytica* (an amoeba parasite) enabling some researchers to resurrect an Archezoa status for either taxa dependant on the direction of transfer [211]. In the published protein tree, *Giardia* clusters strongly (100% bootstrap support) with *Entamoeba*, a relationship for which there is no support from the literature [11]. Roger et al. [211] suggested that this was probably an artifact of analysis, arising from a failure to accommodate between site-rate variation and/or observed amino acid composition inequalities between sequences effectively leading to LBA. Reanalyses of this data, adding a *CPN60* sequence from another diplomonad, *Spironucleus barkhanus* [113], demonstrated the two diplomonads clustering together with *Trichomonas* – as they should (see Box 17.4) – and *Entamoeba* clustering with the aerobic slime mould amoeba, *Dictyostelium*, consistent with other data [11]. These results suggest that the grouping of *Giardia* and *Entamoeba* was an artifact of analysis, most probably a form of LBA and not the result of eukaryote to eukaryote HGT. This analysis also supports the polyphyly of these two branches, providing further evidence against the archezoa hypothesis.

Analyses of the SSU rDNA of the Microsporidia, *Trichomonas*, *Giardia*, and *Entamoeba* (all previously classified as Archezoa) has demonstrated at least one further case of LBA. Initial SSU rDNA eukaryote trees showed the Microsporidia forming the deepest branch of the eukaryotes close to metamonad branches, consistent with the archezoa hypothesis [31,32,232]. This group of obligate metazoan parasites lacks peroxisomes and possesses small prokaryote-like ribosomes, which were interpreted as additional evidence for a primative branching position [31,44]. However, analyses of protein coding genes demonstrated mitochondrial functioning homologues [81,109] (see Box 17.1), suggesting mitochondrial ancestry. Phylogenetic analyses of protein coding genes with correction for differences in site rate variation, demonstrated that the Microsporidia either forms a sister-group to the fungi [110] or is placed within the fungi [131], suggesting that their placement at the base of the eukaryotic tree was an artifact of LBA in the SSU rDNA phylogeny.

The phenomenon of LBA is fundamentally linked to the issue of outgroups. Inclusion of a suitable outgroup and intermediate taxa between the outgroup and the ingroup of interest is essential for the reconstruction of robust, meaningfully orientated phylogenetic trees. The question is thus: What choice of outgroup and/or intermediate taxa will ensure avoidance of the effects of LBA? The answer to such a question is obviously specific to each group of taxa being studied, but from a general point of view, the problem posed by long branches is not so much the length of the branches *per se*, but the fact that the same substitutions have accumulated, not by common ancestry, but by chance along two branches and that rapid sequence evolution has erased all other evolutionary signal due to mutational saturation. Thus, the only way to "shorten" such branches is by including suitable intermediate taxa in an analysis to ensure that such long branches are broken up; see Box 17.6 for a discussion of this topic using the example of trypanosome phylogenies.

17.2.5.2 The effect of adding more taxa The effects of adding more taxa on overall phylogenetic topology are now well recognized. By way of illustration, research into the evolution of parasitism in kinetoplastids, and in particular trypanosomes, illustrates the importance of balanced taxon sampling on phylogenetic relationships and the accuracy of conclusions based on such relationships.

In eukaryotes, the first broad molecular study of evolution was undertaken by Sogin et al. [234] who used phylogenetic analysis of SSU ribosomal RNA sequences from kinetoplastid flagellates. Subsequently, evolutionary analyses became more focused, initially on studies concerning the origins of parasitism in the kinetoplastid [72,140], and latterly on detailed analyses of evolutionary relationships among, for example, *Trypanosoma* and *Leishmania* spp. [42,162,155]. As phylogenetic investigations focused on specific species relationships, the number of species representing each genus in successive studies also increased, with a progression of ideas concerning the evolutionary relationships between the species. This process is well illustrated in evolutionary studies of trypanosomes, where the conclusions of initial SSU ribosomal RNA-based studies

that included only one or two species were gradually altered by inclusion of more taxa in subsequent studies [91,93,243].

For example, an early SSU ribosomal RNA-based tree [163] included three trypanosome species, *Trypanosoma brucei*, *T. cruzi*, and a third species from a fish. In common with many other early studies, this tree indicated that the genus *Trypanosoma* was paraphyletic. Increasing the number of species to seven still left *T. brucei* outside the main trypanosome clade and outside a trypanosomatid clade containing *Leishmania* and *Crithidia* [162]. The inclusion of four more trypanosome species [155] demonstrated for the first time that the genus *Trypanosoma* was, in fact, monophyletic and addition of more outgroup taxa considerably strengthened this result. Subsequent trees included up to 47 trypanosome species [91,243] and unequivocally supported monophyly of trypanosomes. Indeed, it is now generally recognized that the conclusions of many early (1991–1996) SSU rRNA-based phylogenetic studies of trypanosomes (e.g. [72,162,163]) were significantly affected by a combination of insufficient taxa, an imbalance in the spread of included taxa and large intra-generic inter-clade evolutionary rate differences;[8]as alluded to by Stevens et al 2001, the limit of the utility of SSU rRNA sequences for exploring trypanosome evolutionary relationships has probably been reached [244].

In a similar example discussed above, the phylogeny of chaperonin 60 (*CPN60*) gene is sensitive to the addition of a further *CPN60* sequence from *Spironucleus barkhanus* which radically alters the tree topology [113]. Thus, the addition of a single key taxon proved critical to illustrating that the two diplomonads with highly divergent sequences: *G. intestinalis* and *S. barkhanus* are not true archezoa, but, like other eukaryotes, inherited their *CPN60* genes from a common ancestor that once contained the mitochondrial endosymbiont.

17.2.5.3 Hard and soft polytomies Few phylogenetic analyses are perfectly resolved and nodes at which it is not possible to determine the exact branching order of three or more taxa are frequently encountered. Such unresolved nodes are referred to as polytomies and, depending on the quality of the data used, the level of saturation present within the data, the choice of taxa included for analysis, they can be more or less frequent in the final tree.

In many instances, polytomies can be resolved by revision of the underlying alignment, modification of the evolutionary model, such that resolution of branching order between taxa can ultimately be achieved. Such polytomies are referred to as "soft" polytomies and, as described, are the product of a weakness in some aspect of the underlying methodology. In certain circumstances, however, it is apparent that taxa must have diverged from each other within a very short period of evolutionary time, such that although such taxa are obviously different overall, for a particular gene marker, for example, SSU rRNA, insufficient differences are identifiable to enable reliable reconstruction of relationships between them. Alternatively, many differences may be available between taxa, but the differences lack a suitable pattern to be able to define unambiguous relationships; such a situation may arise when nucleotide positions are saturated, for example, third positions within codons of open reading frames, leading to homoplasious changes within sequences. The inability of phylogenetic methods to determine relationships between multiple taxa due to speed of evolutionary change and/or saturation is referred to as a "hard" polytomy.

For example, in some early evolutionary studies of trypanosomatids, ribosomal RNA data did not provide sufficient resolution to permit the exact branching order of key groups to be determined, such that some trees showed polytomies of up to eight taxa [243]. With hindsight and the benefit of now having analyzed alternative classes of gene, for example, *GAPDH*, it seems probable that the limit of the resolving power of the SSU ribosomal marker over the time scale by which many trypanosomes evolved may have been reached [93,243].

17.2.5.4 The effects of rate differences between and within genes Not all genes are suitable for resolving all phylogenetic relationships and terms such as "appropriate level of resolution" are often employed. Ultimately, such *levels of resolution* relate back to the fact that (a) not all genes within a single species evolve at the same rate; (b) homologous genes within closely related taxa do not all evolve at the same rate; and (c) not all regions within a gene evolve at the same rate. For example, in genus *Trypanosoma*, rate differences are apparent between genes, such that different genes and gene classes evolve at many different rates within the genus [242]. Some of the main factors associated with differences in rate between genes include the following:

(i) *Cell location*, that is, in an organelle (e.g., mitochondria or chloroplasts) versus the cell nucleus – some estimates suggest that genes within mitochondrial DNA (mtDNA) evolve at around 10 times the rate of genes in nuclear DNA [29].
(ii) *Coding versus non-coding regions*, that is, ultimately translated sequence versus all classes of non-coding genetic material, including introns and spacer region sequences.
(iii) *Structural effects*, for example, effects of secondary structure within ribosomal RNA genes, whereby substitutions in regions corresponding to loops may evolve rapidly and become saturated, leading to reversals, homoplasy and inaccurate phylogenetic estimations.

Arguably, one of the best illustrations of how molecular clocks become inaccurate over time due to reversals and

[8]In contrast, throughout the period of development and enlargement of SSU rRNA-based phylogenies, studies based on protein-coding genes (e.g. [1,95,96,99]) consistently and unequivocally supported monophyly, a conclusion confirmed by Hamilton et al. [93] in a recent broad-scale analysis of *GAPDH* DNA and protein trees from an extensive range of trypanosomatid taxa [93]. Trypanosome monophyly is further supported by the phylogenies of Simpson et al. [229] based on heat shock protein 90 amino acid sequences.

saturation is the example of mammalian mitochondrial DNA (mtDNA) sequence evolution, whereby the degree of sequence divergence observed between taxa begins to fall below expected levels in more ancient evolutionary relationships [28]. Ultimately, an understanding of such processes can lead to the development of robust molecular clocks able to take account of evolutionary heterogeneity between lineages – see Section 17.4.1.

17.2.5.4.1 The effects of site rate differences Non-model-based methods for constructing phylogenies, for example, parsimony and some methods for calculating distances, assume that each nucleotide position within a sequence is equally likely to undergo a substitution. Such an assumption simplifies calculation of evolutionary relationships, but does not accurately reflect biological reality, as it is well known that average rates of substitution vary widely in different parts of genes and pseudogenes, such that different regions of DNA sequence may have very different probabilities of change. Accordingly, modern model-based methods and programs, for example, ML, incorporate models of rate heterogeneity which assume independent rate variation according to a rate distribution and can include a correction for invariant sites. In such models, the rate at a site, although random, is assumed fixed throughout the evolutionary tree. This is implemented through use of the gamma distribution that specifies the range of rate variation among sites, the precise shape of which is dependent on the ratio of variant to invariant sites in the data; see [280] for details.

The Microsporidia (amitochondrial obligate intracellular parasitic protists) provide a good example of how correcting for differences in site rate variation has allowed a long-running evolutionary debate to be resolved. Initial phylogenetic trees based on SSU rDNA [145] and on elongation factor 1α and 2 (*EF-1α, EF-2*) [100] sequences suggested that Microsporidia diverged early in the tree of life. However, later phylogenies constructed from tubulin [130] *HSP70* [81] and RNA polymerase II [110] placed Microsporidia in the eukaryotic crown, favoring a position either within, or as a sister-group to, Fungi. Clearly both evolutionary scenarios could not be correct. Re-analysis of elongation factor *EF-1α* and *EF-2* sequences (which had previously been taken as support for an early, Archezoan divergence of these protists) showed such support to be weak and likely to have been caused by artifacts in the phylogenetic analyses [110]. Specifically, the re-analysis corrected for rate variation and showed that support for an early divergence of Microsporidia (as opposed to a Microsporidia + Fungi relationship) could be attributed to a failure to account for rate variation.

In addition to this form of site rate variation, which as noted, is now dealt with in most model-based methods by the use of the gamma function and/or correction for invariant sites, work from a variety of studies [77,148,169] indicates that variation in substitution rate may also occur in different lineages within a phylogeny, that is, within different clades within a tree. A number of researchers have now developed software that can be useful in detecting whether different rates occur in two different sub-trees of the larger tree and where these differences occur [198,252] allowing such variation to be identified and dealt with.

Finally, readers should be aware that this short review of forms of site-rate variation is far from complete and a range of other forms of site-rate variation, for example, heterotachy = within site-rate variation through time, have also been documented [151].

17.2.5.4.2 Nucleotide composition bias The potentially problematic effect of compositional heterogeneity in sequence data on phylogenetic inference has been recognized since the early 1990s (e.g. [40,76,78,90], see also Jermiin et al. [126] and refs therein) and it is clear that compositional heterogeneity can mislead methods commonly used to infer phylogenetic trees. It has been suggested that to reduce the effect of nucleotide composition variation, inferred protein sequences rather than DNA sequences should be analyzed [98], the expectation being that compositional bias will be effectively removed at the amino acid level, allowing the correct tree to be recovered. Balanced against this view is the loss of information when converting from DNA to protein [280], together with the observation that amino acid composition biases can also occur in some protein sequences [76].

However, although it is clear that compositional heterogeneity can mislead phylogenetic methods, it is still unclear how much compositional convergence is necessary before the phylogenetic methods fail to recover the correct topology. Recent studies to address this question [40] using Monte Carlo simulations, concluded that "rather extreme amounts of convergence are necessary before parsimony begins to prefer the incorrect tree." Other simulation studies have reached similar conclusions (e.g. [78]), whereas Jermiin et al. [126] found that compositional heterogeneity in sequence data increased the difficulty with which short internal branches could be inferred by parsimony, ML and neighbor-joining methods (with distances calculated using the Jukes-Cantor model of nucleotide substitution). However, they [126] also showed that neighbor-joining, with distances estimated using the LogDet method (developed to deal with heterogeneous nucleotide frequencies), had no difficulty in inferring internal branches under conditions where the three other methods failed; the LogDet method recovered the correct topology nearly every time and it was only when true internal branch lengths were short that the frequency of successful phylogenetic recovery fell below 100%. Overall, such findings reinforce the importance of considering compositional heterogeneity when choosing a method for phylogenetic inference.

Nevertheless, the benefits of using LogDet to overcome the distorting effects of compositional variation are not universally recognized and several studies of empirical data have led to concern about the conditions under which the LogDet method will deal correctly with compositional heterogeneity (e.g. [35,40,75]). For example, some authors have suggested that factors such as rate heterogeneity among lineages [75] and

among sites [40], might also have biased phylogenetic estimates independently of compositional heterogeneity. Moreover, other simulation studies (e.g. [217]) have found that within realistic observed levels of heterogeneity (for mammalian genes), the accuracy of a range of commonly used methods of tree reconstruction (e.g., neighbor-joining, minimum evolution, maximum parsimony, ML) is comparable to those simulated with homogeneous nucleotide frequencies. Significantly, the LogDet distance method performed no better than distance methods that assume substitution pattern homogeneity among sequences, suggesting no significant relationship between phylogenetic accuracy and substitution pattern heterogeneity among lineages, even when taxon sampling was increased [217].

17.2.5.5 Gene expression Because genes are not organisms, there is an inevitable logic flaw in assuming that genetic evolution is necessarily the same as the evolution of the organisms from which they were derived. However, this is not due just to the questions of sample identity, alignments, or choice of sequence and sample size mentioned above – it is also reflected in the increasing evidence that evolution does not proceed only by sequence changes. Gene rearrangement [52,57,275] and HGT (see Box 17.1 for example) may be significantly more important than point mutations as sources for evolutionary change. Moreover, gene expression, that dictates what an organism is and does, can be controlled by another gene or group of genes – or, in at least some cases, by heritable factors that may not be represented in the sequence code. Such factors may involve conformational changes, revealed for example as heterochromatin that can affect which genes are transcribed in a particular specimen [61]; see also http://www.euchromatin.net/. They may also involve replicable proteins, such as prions, that again influence gene expression [180]. Such epigenetic factors are poorly understood, but, as they can be heritable, they may be important evolutionary features affecting homology between higher biological characteristics without affecting the underlying DNA sequences.

17.3 METHODS OF COMPARING PHYLOGENIES

17.3.1 Methods of Assessing Congruence Between Phylogenies

Relationships between phylogenetic trees can be formally assessed using a range of congruence tests to provide a statistical value to indicate whether or not two genes produce the same phylogenetic tree, that is, are congruent. These include*** the incongruence length difference (ILD) [65], Templeton [260], Kishino–Hasegawa (K–H) [135], Shimodaira–Hasegawa (S–H) [226], and the approximately unbiased (AU) test [224], where most of these statistical tests are available in the program CONSEL [225]. The use and appropriateness of these tests have been the subject of many studies [43,85,108,240], though, as yet, there appears to be no consensus as to which, if any, of the tests are best at assessing phylogenetic congruence. For example, Goldman et al. [85] suggested that the appropriateness of the K–H test should be carefully considered when comparing topologies because the test is only valid when the topologies being compared are specified *a priori*. Unfortunately, this means that the K–H test may be severely biased in many cases in which it is now commonly used and, moreover, the S–H or the AU test will produce more accurate results when using ML. Simulation studies have shown that in some scenarios, the AU test is more appropriate, as unlike the S–H test the AU test is not compromised by increases in the number of trees compared. However, in some cases the S–H test is better when the trees compared are nearly equally as good [224].

The ILD test is described as an inaccurate method for indicating topological congruence by Barker and Lutzoni [13] because factors including transition: transversion ratio bias, base composition and branch lengths may cause the results to be bias. However, Hipp et al. [108] concluded that a comparison of results produced by the ILD test in conjunction with the K–H, S–H, and Templeton tests should give an accurate overall result. Cunningham [43] compared the Templeton and Rodrigo [207] tests with the ILD test and showed that the ILD test is most accurate and suggested that invariant characters should be removed.

Overall, there does not appear to be a distinct trend in the pattern of variation between the various tests. As described, the inaccuracies and inconsistencies in many of the available statistical tests have been the subject of numerous papers [43,85,108,224,240], each of which identify potential problems in the different tests without reaching a consensus on which tests are the most accurate overall.

17.3.2 Methods for Studying Coevolution

17.3.2.1 Coevolutionary considerations Although the phylogeny of any organism must be considered in relation to its environment and the selective and evolutionary forces within such an environment, this requirement is perhaps even more poignant in parasite phylogenies, where phylogeny is often intimately linked to host and where host switching and, "arms race" interactions and resource tracking may have acted to affect parasite evolution over time. Since the landmark study of Hafner and Nadler [92], molecular data have become widely used in the study of host–parasite coevolution, and techniques for comparing host–parasite phylogenies are now well developed, for example, the program TREEMAP [37]. Although early methods for studying parasite evolution relied on morphology-based phylogenies and methods derived from biogeography, molecular data have now provided the opportunity to test co-speciation using genetic markers evolving by, potentially, the same processes or under co and/or counter selection in both parasite and host, that is, sequences from homologous genes, or genes coding for interacting products, that is, in a genetic "arms race" scenario [45].

17.3.2.2 Methods for exploring coevolution

Computational aspects of host–parasite phylogenies form part of a set of general associations between areas and organisms, hosts and parasites, and species and genes. Within each of these associations, one lineage is associated with another, and can be thought of as tracking the other over evolutionary time with a greater or lesser degree of fidelity [189]. The problem is not new, and certainly the commonalities of exploring vicariance biogeography (organisms tracking areas) and host–parasite co-speciation (parasites tracking hosts) have been recognized for some time [25,216].

Consequently, methods for comparing host–parasite phylogenies are now well established and fall into two basic categories, defined in terms of the way the data are interpreted in relation to the comparison of host–parasite phylogenies: *a posteriori*, for example, Brooks' Parsimony Analysis (BPA) [26], or *a priori*, for example, reconciled trees and other model-based methods, as implemented in, for example, the programs COMPONENT [181,182] and latterly, TREEMAP [37]. As outlined below, the relative merits of the two philosophies inherent in these two approaches remain hotly debated [27,55,56,187,188].

17.3.2.3 Philosophies underpinning methods for studying host–parasite phylogenies

17.3.2.3.1 BPA

BPA is based on the assumption that there need not be any model-like regularities in phylogenies and that (co)evolutionary processes are so contingent on history, for example, vicariance events, that no *a priori* model will be sufficient for capturing all the relevant detail [27]. Unlike model-based methods – its proponents suggest – it does not seek to maximize fit to any predetermined hypothesis, but instead offers a framework to ask such questions as: How many co-speciation patterns that do exist are due to mutual modification leading to mutual speciation, and how many are simply byproducts of vicariant speciation? Ultimately, however, lack of an explicit underlying model precludes formal testing and, consequently, BPA will not be considered further in this chapter.

17.3.2.3.2 Reconciled trees, maximal co-speciation and event–cost methods

An alternative approach to BPA is to map one tree (that of the parasite/pathogen) onto another tree (that of the host); a range of event–cost model-based methods designed to maximize co-speciation [86,186] can then be used to reconcile differences in the patterns between the host and parasite evolutionary trees. The philosophy of the maximum co-speciation approach is defined by Page [186] as follows: "Cospeciation is joint cladogenesis of host and parasite. If we regard host cladogenesis as the primary cause of cladogenesis in the parasite, then the host phylogeny is the "independent" variable and the parasite phylogeny is the "dependent" variable. The host phylogeny explains the parasite phylogeny to the extent that speciation events in the parasite phylogeny are co-speciations. Hence as natural criterion for choosing a reconstruction is maximizing the extent of co-speciation, that is, the ability of the host phylogeny to explain the parasite phylogeny."

Computer methods based on reconciled trees and maximal co-speciation have been developed since the early 1990s (see [186,241] for recent comprehensive reviews), successively incorporating the ability to deal with associated issues, such as host switching (e.g., COMPONENT [182,183] and TREEMAP [37]). However, due in part to the optimality criteria used in some earlier programs, it was apparent that many reconstructions could imply the same number of co-speciation events, thus yielding multiple solutions. This problem was addressed by the development and inclusion of cost-event-based analyses [36,213,214] that consider and evaluate each hypothesized past association individually, to find the least-costly solution (e.g., TreeFitter [214] and Jungles [36] in TREEMAP 2.02). Indeed, the approach is exemplified by the most recent implementation of the program TREEMAP 2.02 [37] that attempts to explain observed relationships by producing a set of solutions that range from those that maximize co-speciation to those that include a minimum of co-speciation enforced by logical consistency – that is, TREEMAP 2 does not simply use co-divergence alone as an optimizing criterion for evaluating solutions. Rather, solutions are based on an *a priori* model that allows the user to define event–cost assignments for a range of different potential events, for example, extinctions, lineage duplications, host switching, to explain the observed pattern of host–parasite relationships (see below).

17.3.2.4 Gene phylogenies versus species phylogenies: a further complication

Early approaches to the study of host–parasite coevolution relied on the construction and interpretation of morphology-based phylogenies, using methods influenced by the allied discipline of biogeography. However, since the study of Hafner and Nadler [92], molecular data have become increasingly more widely used in the study of host–parasite coevolution. Significantly, molecular data have provided the opportunity to test co-speciation using genetic markers evolving by, theoretically, the same processes in both parasite and host, that is, sequences from homologous genes, or genes coding for interacting products, that is, those involved in an "arms race" system. However, as lice have shown [191], mitochondrial DNA evolves considerably faster than vertebrate host DNA and has different substitution characteristics that are shared with other insects. Indeed, based on what is known about molecular clocks and the variability in clock speeds between different genes, even within a single species [242,281] the use of homologous gene sequences or "interacting" genes is essential. Thus, when comparing molecular phylogenies of a single parasite gene with a phylogeny derived from a single host gene, the potential for a lack of parity between the single gene trees and the species trees must not be overlooked. If molecular host–parasite phylogenies are not congruent, it may be due to the particular evolutionary

history of one or other (or both) of the two gene phylogenies, and not necessarily to a lack of congruence in species phylogenies. Of course, lack of congruence could be due to genuine host–parasite evolutionary incongruence as a result of host switching or other evolutionary scenarios such as speciation independent of host, parasite extinction, noncolonization of all host lineages, or failure to speciate with host [184]. Ultimately, however, the limitations of single gene phylogenies are well known and rarely, if ever, equate perfectly to overall species phylogeny; consequently, host–parasite systems might thus be thought of as incorporating limitations from multiple sources, that is, from both the host and the parasite sides of the system under study (= two complete genomes). It remains to be explored whether this means that those host–parasite phylogenies that are seen to be congruent should be viewed with increased significance (however one chooses to define "significance"). Moreover, does phylogenetic analysis of homologous genes in both parasite and host, which may (or may not) be under similar biological constraints in both, mean an *a priori* increased likelihood of evolutionary congruence? The answer to this last particular issue may not be known until patterns of coevolution have been evaluated for a broad range of host–parasite systems with a range of ecologies, based on a broad range of homologous and nonhomologous molecular markers.

17.3.2.5 Computational resources

17.3.2.5.1 Statistical tests of congruence/incongruence

A number of tests to perform straightforward assessments of congruence/incongruence between tree topologies may be used to explore relationships between parasite and host phylogenies. These include the K–H [135] and ILD [13] tests that are used to assess phylogenetic homogeneity of DNA sequences and combinability of data from different sources. Both are implemented in the current version of PAUP [254]. However, statistical tests developed specifically for assessing congruence between parasite and host phylogenies are also available [119,144].

Huelsenbeck et al. [119] proposed two tests to examine the null hypothesis that host and parasite trees are identical, based on phylogenetic estimates obtained using either ML or maximum posterior probability (i.e., Bayesian inference).

The ML approach uses a likelihood ratio test to examine the null hypothesis, H_0, that the host and parasite trees are identical, H_1 being that the host and parasite trees are not identical. Under H_0, a likelihood value is calculated under the constraint that host and parasite phylogenies are identical, but allowing the parameters of the substitution model to differ. Similarly, a likelihood value is calculated under H_1,** in which the host and parasite trees are not constrained to be identical. The degree of congruence (or otherwise) between the two phylogenies is then assessed by calculating the ratio of the observed ML values: ML H_0/ML H_1. The significance of the observed ratio is then assessed by parametric bootstrapping in which simulated data sets are generated under the assumption that the H_0 is correct; the observed ratio can be compared and assessed against a chosen significance level, for example, 95%.

The Bayesian analysis uses a conditional probability test to test for identical (or otherwise) tree topologies. The Bayesian approach directly calculates the probability that the host and parasite trees are correct [118]. This is achieved, by calculating the posterior probabilities of the host phylogeny – given the data – and the parasite phylogeny – given the data. The probability that host and parasite phylogenies are in agreement (congruent) is the sum of the posterior probabilities for all possible congruent pairs of host and parasite topologies. The probability of each phylogeny is calculated by Bayesian inference using an appropriate program, for example, MrBayes [215].

More recently, Legendre et al. [144] have developed ParaFit, a matrix permutation test of co-speciation. ParaFit aims to test the significance of a global hypothesis of coevolution between parasites and hosts. To statistically assess the hypothesis of host–parasite coevolution, ParaFit combines three types of information: the phylogeny of the parasites, the phylogeny of the hosts, and the observed host–parasite associations. Each phylogeny is described by a matrix of patristic distances among the species along each tree, which in turn are transformed into a matrix of principal coordinates [142,144]. The null hypothesis, based on the two phylogenetic trees and the set of host–parasite association links, is that evolution of the hosts and parasites has been independent, that is, that one is random with respect to the other. ParaFit also allows the significance of each individual host–parasite link contributing to the overall relationship to be considered and estimated. ParaFit is available at http://www.fas.umontreal.ca/biol/legendre/.

17.3.2.5.2 TREEMAP TREEMAP is a state-of-the-art program based on the reconciled tree approach. The key difference from COMPONENT (from which it was developed) being the ability to incorporate host switching as an explanation of the observed pattern of host–parasite associations.

Its current implementation is TREEMAP version 2.02 [37], a program that also provides an option to implement the Jungles [36] event–cost method to find all potentially optimal solutions to explain observed patterns of host–parasite association. The implementation of Jungles in TREEMAP 2.02 avoids many of the problems associated with the use of optimality criteria in some earlier programs, where there could be many reconstructions implying the same number of co-speciation events, thus yielding multiple solutions. TREEMAP 2 avoids this problem by using Jungles to search for all feasible reconstructions within bounds set by the user. Significantly, the Jungles algorithm in TREEMAP 2 can explore all switches, including those that require subsequent sorting events to ensure that source and destination are contemporary (known as "weakly incompatible switches"). To evaluate individual reconstructions, the user can specify costs for each event (duplication, host switch, and sorting events). In this way, the user can still explore alternative reconstructions (as in previous

programs), but not be swamped with many similar, but nonoptimal solutions. Jungles uses four parameters to calculate the overall cost of each hypothesized past association individually; these are co-speciation, duplication, lineage sorting, and host switching. TREEMAP 2 then estimates the significance of observed codivergence, total cost, or the number of another event type, using randomization tests.

17.4 DATING PHYLOGENETIC TREES

17.4.1 Molecular Clocks

A key feature of molecular phylogenies is the idea that not only can relationships be revealed, but that divergences can also be dated using various models of the expected rate of accumulation of mutations. Again, the underlying theory is sound, but is subject to a number of assumptions that can be difficult to sustain. Chief among these is the notion that within a given gene, substitutions become fixed at a constant rate, according to a Poisson distribution and equivalent to the underlying rate of mutation – this is the "neutral theory" first proposed in the late 1960s [133]. For a given gene, variations in evolution rate between species are explained by lineage effects, which account for differences in mutation rates between species based on factors such as generation time, metabolic rate, efficiency of DNA repair, number of DNA replication events in germ line cells, and exposure to mutagens, of which the effects of the first two are most well documented, for example [146,157].

Adherents to the neutral theory would argue that for a given gene, one or more of the lineage effects would be sufficient to account for differences in rates of evolution observed between different taxa. However, based initially on observations that the rate of amino acid substitutions in various proteins was not consistent with a Poisson distribution, the neutral theory was extended to become the "nearly neutral theory," to take account of the fact that both drift and selection also appear to be important for determining nucleotide substitution rates. Today, the theory incorporates the idea that nearly neutral mutations may be slightly deleterious or slightly advantageous, and appears to offer at least some explanation for variation in non-synonymous substitution rates.

From a phylogenetic point of view, an understanding of molecular clocks is vital when attempting to interpret the timing of divergence events from phylogenetic trees. Perhaps the single most important factor is the ability to determine whether or not a given gene is evolving in a clock-like manner, such that divergence events can be related to evolutionary time. Several tests are available to assess adherence of molecular data to a uniform clock: the dispersion index, the relative rate test and, of particular relevance to modern phylogenetic analysis methods, the likelihood ratio test [117]. However, different methods of phylogenetic analysis are not equally affected by such factors such as lineage effects, leading potentially to distortion of relationships. For example, parsimony methods may be particularly affected by unequal rates of evolution within lineages, resulting in LBA and inaccurate recovery of true evolutionary relationships [67], whereas even model-based methods can be affected if the model does not incorporate a suitable gamma function to account for heterogeneity of evolutionary rates across different sites within sequences [223]. A number of well-documented likelihood-based tests are now available to test whether or not defined lineages or clades are adhering to a molecular clock, for example, likelihood ratio test, relative rate test; see [196] for further details.

Nevertheless, in the field of parasitology and pathogen research, phylogenetic trees have increasingly been used as a basis for dating divergence events. In many instances, the timing and antiquity of divergences have been calculated by assuming a molecular clock, though rarely has the validity of assuming a uniform clock been tested. For example, in trypanosomes, the use of (probably inappropriate) clocks led to an extremely wide and somewhat improbable range of divergence estimates being presented, for example, trypanosomes/Salivarian trypanosomes: 300 million years (MYs) [91], trypanosomes/*Leishmania*: 340 MYs [72], *T. cruzi*/*Trypanosoma rangeli*: 475 MYs [23]. Thankfully, this practice now appears to be changing [24,128].

In the case of trypanosomes (genus *Trypanosoma*), confirmation that the genus is monophyletic (as supported by findings from a variety of protein gene phylogenies [1,3,93,96]) has allowed the construction of sufficiently robust phylogenies [172] to facilitate exploration of the different rates at which lineages within trees are evolving. However, certain taxa within the group, for example, *T. brucei*, display marked differences in rates of nucleotide substitution in SSU rRNA sequences between clades [178] and high levels of homoplasy [155]; for example, the substitution rate in *Trypanosoma vivax* (a parasite of cattle in Africa and South America) is up to three times that in other closely related lineages [91]. Thus, different evolutionary rates exist between trypanosome clades, but related taxa appeared to be evolving at locally (in terms of phylogenetic proximity) similar rates, suggesting that early SSU-based studies may have been confounded by one or more of the following: variable substitution rates, simplistic models of molecular evolution, and a lack of suitable taxa to break up long branches.

Accordingly, Stevens and Rambaut [242] used the program RHINO [198] to estimate relative rates of substitution between specified lineages within trypanosome trees, that is, to estimate local clocks, within a ML framework; several studies of local clocks have also been reported previously [75,199,281]. Clades to which rate parameters were assigned were specified *a priori* on the basis of independent biological knowledge, allowing a hierarchy of competing models of rate heterogeneity to be tested using likelihood ratio tests. Results showed that within the genus *Trypanosoma*, SSU rRNA genes were evolving at at least[9] four significantly different rates,

[9]Even the four rate model was rejected when compared to a non-clock model [242], suggesting that the dataset was evolving at five or possibly more significantly different rates.

each of which was associated with a particular phylogenetic clade. Of these clades, the predominantly African mammal-infective Salivarian trypanosomes were evolving at more than four times the rate of other trypanosome species, with *T. vivax* evolving at more than twice that of other Salivarian trypanosomes and approximately 7–10 times the rate of non-Salivarian trypanosomes. Reasons for such rapid evolution in this single taxon are unclear and, whereas the assumption of sequence homogeneity within rRNA genes is widely accepted because concerted evolution is thought to act in all eukaryotes, there is a growing list of examples, including several *Trypanosoma* species, where complete homogeneity is not the case [250,251]. Accordingly, the possibility that the single *T. vivax* SSU rRNA sequence employed in phylogenetic analyses is either a pseudogene, or another form of a divergent copy which is not being homogenized by concerted evolution, cannot be ruled out. Whatever the reason, its rRNA molecular clock appears to be running at a very different speed from other Salivarian trypanosomes.

Clock-based evolutionary studies of *T. cruzi* rRNA sequences have also been performed and suggest that the two major lineages of *T. cruzi*, I and II [6], diverged between 16 and 18 MYs [156] or 37 and 84 MYs [23] ago, depending on the clock speed assumed. Again, evidence from protein-coding gene studies [128] can be used to shed additional light on these clock-based dates, in this case suggesting that the most recent time frame (or indeed an even earlier divergence) may be the most plausible. Certainly, a cytochrome *b*-based divergence estimate of 10.6 ± 2.1 MYr [24], accords well with biological theory [222] and estimates based on biogeographical data [244]. Likelihood ratio analyses of evolution rates within a number of *T. cruzi* genes, namely SSU rRNA, the D7 region of 24Sα rRNA and mitochondrial cytochrome oxidase *b*, have also shown that across all major lineages within this species, at least for these genes, rates of evolution are not significantly different, that is, these genes are each evolving in a uniform clock-like manner within *T. cruzi* [24,128].

Thus, in trypanosomes, whereas (in at least some species) evolution appears to be proceeding according to a single molecular clock, broader clades comprising similar trypanosome taxa (on the basis of biology, ecology, and phylogeny) are apparently evolving at significantly different evolutionary rates.

17.4.2 Biogeography and Fossils

Interpretation of phylogenetic trees in relation to other events in evolutionary time depends on conversion of branch points into dates to estimate time of divergence of different clades.

The molecular clock approach (see Section 17.4.1) assumes that changes in a given sequence accumulate at a constant rate, and thus that the difference between two sequences is a measure of the time of divergence. From a post-genomics standpoint the simplicity of some of these notions look almost quaint and, indeed, the approach has been amply discussed and criticized over the years [133,146] – see above. A second way in which times of divergence can be estimated relies on congruence of host and parasite phylogenies. Thus, parasite trees can be calibrated by reference to known time points within host phylogenies (see Section 17.3.2), which have been independently dated from the fossil record. This assumes that existing associations of hosts and parasites reflect past associations – which may or not be true.

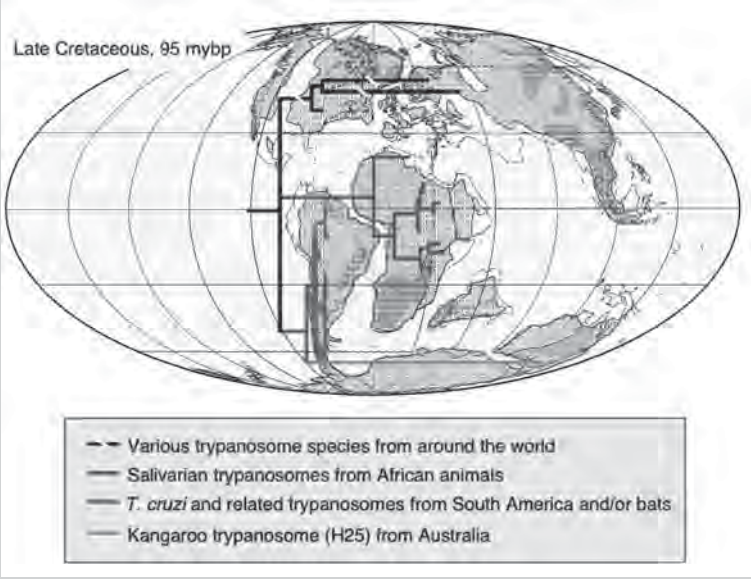
Thirdly, divergence events can be dated by reference to known biogeographical events. This approach to phylogenetic calibration is known as vicariance biogeography [273], and several studies of trypanosomatid evolution have drawn on this technique. For example, using the breakup of Africa and South America to date the divergence of *Leishmania* and *Trypanosoma* [140], to corroborate the split between Old and New World *Leishmania* [72], and to date the divergence of *T. brucei* and *T. cruzi* [243]. In this latter study [243], the composition of host taxa in clades defined in a trypanosome phylogeny was used to date the divergence of the Salivarian trypanosome clade to around 100 million years before present (mybp), when Africa became isolated from the other continents (Box 17.7). This was based on the observations that the *T. brucei* clade consisted exclusively of African mammalian tsetse-transmitted species and that trypanosome species from African amphibia and reptiles are unrelated; a previous study considering only palaeoecological data arrived at a similar dating [141]. The composition of the *T. cruzi* clade – mostly mammalian trypanosome species from South America – also agreed with this interpretation, whereas the inclusion of an Australian kangaroo trypanosome in the clade reinforced the idea that evolutionary diversification of *T. cruzi* and relatives occurred within the New World; the split of South America from Antarctica and Australia is thought to have occurred later than the separation from Africa [41] (see Box 17.7).

Occasionally, it is also possible to date nodes within phylogenies precisely by obtaining sequence data from DNA extracted from preserved biological tissue. In the case of pathogens and parasites, DNA is usually extracted from preserved diseased tissues or cadavers, for example, molecular characterization of the influenza virus responsible for the 1918 "Spanish flu" pandemic, using autopsy lung tissues from flu victims [257], and characterization of *Mycobacterium tuberculosis* from Egyptian mummies [282]. Using a similar approach, it is also possible to explore the evolution of very rapidly evolving pathogens, for example, HIV, by characterizing isolates taken at specific time points in the progress of the disease, even within a single patient [112]. Such studies may prove vital in elucidating the patterns and mechanisms by which pathogens evolve and are able to avoid not only the host's own immune mechanisms but also vaccines and targeted drug therapies.

Several studies have demonstrated that HGT occurs between plant mitochondrial genomes across established species/mating barriers. In some cases HGT occurs in both directions between host and parasitic plant lineages [46] consistent with the hypothesis that HGT occurs between host and parasites that form close associations [203]. In the case of the *Amborella* the HGT is considerable with 20 of 31 known

BOX 17.7 – TRYPANOSOME BIOGEOGRAPHY

A partial trypanosome phylogeny superimposed on a map of the world in the Late Cretaceous period, approximately 95 mybp. Stevens et al. [243] used this approach, together with information on the origins (host and geographical) of trypanosomes in clades defined in their phylogeny, to date the divergence of the Salivarian trypanosomes from *T. cruzi* and other trypanosomes to around 100 mybp, when Africa became isolated from the other continents. After, Cox [41], Smith et al. [284] and Stevens et al. [243] (biogeographical relationships shown are at the continental level, branches within the continents do not indicate local biogeography). See color plates.



mitochondrial genes derived from HGT from different plant genomes [17]. The phylogenetic data used to support these cases of HGT rests on contradictions in tree topologies between species trees, supported by morphological and phylogenetic data with contrasting gene trees showing hypothetical HGTs. In addition, Mower et al. [173] demonstrate in their analyses that the observed incidences of plant–plant HGT are not an anomaly of hidden paralogy or inappropriate taxon sampling. The analytical methods reported are comprehensive and reliable (accounting for site rate heterogeneity) suggesting that combined with the extensive number of transfers reported this is a relatively common phenomenon [16,17,46,276]. In 2003, Won and Renner [276] demonstrated a HGT leading to the replacement of a group II intron and exon b and c of the *nad1* subunit from the mitochondria of angiosperms plant species to the unrelated Gnetum plant lineage. This HGT can be tracked down to the last common ancestor of the Southeast Asian lineages, suggesting that this particular HGT is a biogeographical marker for the spread of this plant species across Asia and can be contrasted against other mitochondrial data sets to compare patterns of biogeography and plant colonization.

17.5 CONCLUSION

Ultimately, the ability to control the diseases caused by pathogens and parasites depends on (a) the ability to recognize the etiological agents of disease, and (b) the ability to maintain long-term surveillance to monitor for the reoccurrence of such agents. Recognition of disease agents depends on the ability to accurately and definitively characterize pathogens and parasites, using appropriate microscopical, serological, biochemical, and molecular techniques, which, in turn, allow populations of disease agents to be tracked, identified to source and monitored.

As the large differences in, for example, pathogenicity and drug resistance and susceptibility of otherwise closely related populations of parasites and pathogens becomes increasingly apparent, so the need to identify parasite and pathogen populations to an ever finer level also increases. Accordingly, phylogenetic techniques are playing an increasingly large role in distinguishing and tracking populations/strains of disease agents. Significantly, unlike, for example, some simple "yes/no" serological typing techniques, phylogenetic characterization has the added

advantage of also being able to provide information on the relative relatedness of different parasite/pathogen populations, facilitating an understanding of how a disease agent evolved, and what might be next in line to pose a similar threat.

Thus, far from being a purely scientific exercise, characterization of parasites and pathogens by phylogenetic methods has become a key tool in the discipline of molecular epidemiology. Likewise, even the often maligned discipline of taxonomy is seen to be crucial in providing a framework (albeit a sometimes rapidly changing framework [38,87]) to underpin, for example, accurate characterization of disease agents prior to vaccine development [51]. Of course, molecular techniques are not the only methods available and, moreover, we must be careful to ensure that the final objective – the ability to reliably recognize disease agents – is kept firmly in focus as the most important factor. As Holmes and Zanotto [111] point out, the genotyping of disease agents should not become a dominant aim of molecular epidemiology as the existence of different genotypes does not necessarily imply phenotypic – in this case, medical – importance. Similarly, from the purely taxonomic point of view, an overreliance on molecular characterization alone may also be detrimental to our understanding of the relationships between parasites and pathogens; as witnessed by a recent debate on the virtues of DNA taxonomy [101,147,258], it would be a "step backwards for science" to replace existing classifications with a system in which only an infinitesimally tiny fraction of an organism's genome is sequenced [147].

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GLOSSARY

Alignment: The arrangement/re-arrangement of nucleotides or amino acids in homologous molecules to maximize similarity or minimize the number of inferred changes among the sequences.

Bayesian analysis: Bayesian methods use a branch of logic applied to decision making and inferential statistics that deals with probability inference, that is, using the knowledge of prior events to predict future events; thus, according to Bayesian logic, the only way to quantify a situation with an uncertain outcome is through determining its probability. Based on probability theory, the theorem defines a rule for refining an hypothesis by factoring in additional evidence and background information, and leads to a number representing the degree of probability that the hypothesis is true. Developed by Thomas Bayes (an English clergyman and mathematician) in the 1700s, it has become increasingly popular since the 1980s with the increasing availability of computer power.

Bootstrapping: A statistical method based on repeated random sampling *with replacement* from an original sample to provide a collection of new pseudoreplicate samples, from which sampling variance can be estimated.

Cladogram: A tree that depicts inferred historical branching relationships among entities. Unless otherwise stated, the depicted branch lengths in a cladogram are arbitrary; only the branching order is significant. See *phylogram*.

Clock speed: A value for relative rates of mutational change.

Congruence: Agreement among data or data sets.

Gamma distribution: The gamma distribution is a continuous probability distribution and is used to describe the distribution of among site rate variation present in sequence data, thereby allowing appropriate corrections for multiple substitutions to be made in phylogenetic methods (likelihood-based methods), which incorporate a gamma distribution parameter. Computationally, the gamma distribution is particularly useful because a single parameter (the shape parameter, α) continuously alters the character of the distribution.

Gene tree: A branching diagram that depicts the known or (usually) inferred relationships among a historically related group of genes or other nucleotide or amino acid sequences.

Heuristic search method: Any analysis procedure that does not guarantee finding the optimal solution to a problem (usually used to obtain a large increase in speed over exact methods).

Homology: Similarity by common ancestry of two or more genes or gene products.

Homoplasy: A collection of phenomena that leads to similarities in character states for reasons other than inheritance from a common ancestor. These include convergence, parallelism, and reversal.

Horizontal gene transfer (*HGT*): Any process in which an organism transfers genetic material (i.e., DNA) to another cell that is not its offspring. By contrast, vertical transfer occurs when an organism receives genetic material from its ancestor, for example, its parent or a species from which it evolved.

Ingroup: An assumed monophyletic group, usually comprising the taxa of primary interest.

Maximum likelihood: A criterion for estimating a parameter from observed data under an explicit model. In phylogenetic analysis, the optimal tree under the maximum likelihood criterion is the tree that is the most likely to have occurred given the observed data and the assumed model of evolution.

Maximum parsimony: A criterion for estimating a parameter from observed data based on the principle of minimizing the number of events needed to explain the data. In phylogenetic analysis, the optimal tree under the maximum parsimony criterion is the tree that requires the fewest number of character-state changes (which may be differentially weighted across characters and/or character-states). Often simply referred to as parsimony.

Monophyletic: A group of taxa derived from a single ancestor, which includes all of its descendants and no other taxa (see also *paraphyletic* and *polyphyletic*).

Neighbour-joining: An heuristic search algorithm for finding a minimum evolution tree.

Non-synonymous substitution: A nucleotide substitution that does result in an amino acid replacement.

Optimality criterion: A function that defines how well data fit a particular hypothesis (as, for instance, a particular phylogenetic tree).

Orthologue: Two homologous genes from different species are orthologous if their most recent common ancestor did not undergo gene duplication.

Outgroup: One or more taxa assumed (usually on the basis of independent biological information) to be phylogenetically outside the *ingroup* that are used as a rooting point of a phylogenetic tree and may be used to assign the direction of change to character-state transformations.

Paralogy: Homology that arises via gene duplication.

Paraphyletic: A group of taxa that contains an ancestor and all of its descendants, but which also contains taxa not derived from the most recent common ancestor (see also *monophyletic* and *polyphyletic*).

Phylogeny: The historical relationships among lineages of organisms or their parts (e.g., genes).

Phylogram: A tree that depicts inferred historical relationships among entities. Differs from a cladogram in that the branches are drawn proportional to the amount of inferred character change.

Polyphyletic: A group of taxa derived from two or more distinct ancestral lineages – a group comprising taxa descended from two or more different ancestors (see also *monophyletic* and *paraphyletic*).

Polytomy: A node in a tree that connects three or more descendent branches. A polytomy (synonym: multifurcation) may represent a lack of resolution because too few data are available for inferring the phylogeny (in which case it is said to be a soft multifurcation) or it may represent the hypothesized simultaneous splitting of several lineages (in which case it is said to be a hard polytomy).

Synonymous substitution: A nucleotide substitution that does not result in an amino acid replacement.

Transition: A nucleotide substitution from one purine to another purine (e.g., A to G), or from one pyramidine to another pyramidine (e.g., T to C).

Transversion: A nucleotide substitution from a purine to a pyramidine (e.g., A to C), or *vice versa* (e.g., T to G).

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CHAPTER 18

Parasites that Manipulate Their Hosts*

Frédéric Thomas,[1] Janice Moore,[2] Robert Poulin,[3] and Shelley Adamo[4]

[1]*Génétique et Evolution de Maladies Infectieuses GEMI/UMR CNRS-IRD 2724, Equipe: "Evolution des Systèmes Symbiotiques", IRD, 911 Avenue Agropolis, B.P. 5045, 34032 Montpellier Cedex 1, France*

[2]*Department of Biology, Colorado State University, Fort Collins, CO 80523, USA*

[3]*Department of Zoology, University of Otago, Dunedin, New Zealand*

[4]*Department of Psychology, Dalhousie University, Halifax, Canada*

18.1 INTRODUCTION

How a parasite (or its offspring) moves from one host to the next is a central topic in parasitology (see Tables 18.1 and 18.2). Understanding such strategies is at the heart of applied aspects of parasitology such as epidemiology and medicine, but it is also important for resolving more basic biological questions. One strategy of transmission that is especially fascinating is that of host manipulation, which occurs when a parasite enhances its own transmission by altering host behavior or host morphology. Parasites of all kinds have been shown to modify these phenotypic traits of their hosts in ways that appear to enhance the parasite's chances of completing its life cycle. From an evolutionary point of view, these changes are compelling illustrations of the "extended phenotype" concept proposed by Dawkins [31], in which genes in one organism (i.e., the parasite) have phenotypic effects on another organism (i.e., the host). From a medical or veterinary point of view, these phenotypic alterations can also be viewed as an expression of the parasite's virulence. Although more subtle than the gross pathology associated with many parasitic diseases, manipulation of host phenotype is nonetheless harmful to the host.

This chapter has three aims. Using well-documented case studies, we will (1) present an overview of the "manipulation hypothesis," (2) discuss the potential of this hypothesis for providing novel insights into the mechanisms regulating individuals at the organismal and ecological levels, and (3) cover several contentious issues related to this hypothesis.

18.2 HISTORICAL OVERVIEW

According to the manipulation hypothesis, a parasite may be able to alter the behavior of a host for its own selective benefit, usually by enhancing its transmission rate. The hypothesis states that such behavioral modification results from *natural selection* favoring enhanced parasite transmission; it is not simply a sporadic byproduct of other physiological activities of the parasite. Early in the twentieth century, scientists began to suspect that parasites could manipulate their hosts (e.g. [27]). The first field evidence came from cormorants *Phalacrocorax carbo* (definitive hosts), which frequently caught fish that were intermediate hosts of the cestode (worm) *Ligula intestinalis*, whereas fishermen were more likely to capture uninfected fish [151]. In laboratory experiments, Bethel and Holmes [13,14] showed that crustacean amphipod (*Gammarus lacustris)* intermediate hosts of acanthocephalans (worm) (*Polymorphus paradoxus*) behaved abnormally; these behavioral changes resulted in increased predation from ducks (definitive hosts). Since the 1970s, studies of behavioral changes in parasitized animals and the enhanced transmission that frequently accompanies these changes have increased. The phenomenon is not only inherently interesting (witness any one of a number of science fiction stories with manipulated behavior as a central theme), but it also demonstrates the ubiquitous importance of parasites to a broader community of scientists. A wide range of parasites are now known to alter host behavior (see [8,25,26,95,114] for reviews]). These studies have shown that parasites can modify a large range of

*Adapted from [136].

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

TABLE 18.1. Major Groups of Protozoan Parasites of Metazoan Hosts, with Representative Genera (from [19])

Parasite taxon	Representative genera
Phylum Microsporida	*Nosema, Encephalitozoon*
Phylum Sarcomastigophora	
Subphylum Mastigophora	*Trypanosoma, Leishmania, Giardia*
Subphylum Opalinata	*Opalina*
Subphylum Sarcodina	*Entamoeba, Naegleria*
Phylum Apicomplexa	
Class Sporozoasida	
Subclass Gregarinasina	*Gregarina*
Subclass Coccidiasina	*Plasmodium, Toxoplasma, Cryptosporidium, Eimeria*
Subclass Piroplasmasina	*Babesia, Theileria*
Phylum Ciliophora	Ciliates

host behavioral traits in ways that can vary in magnitude, from slight changes in activity levels to the production of complex and spectacular behaviors ([95,119], see examples in Section 18.3).

As in many other fields, the early thrill of discovery and ready explanation has later been modulated by circumspection and caution. By the late twentieth century, scientists were bringing more rigor to the manipulation hypothesis, demanding more evidence for the adaptive nature of a behavioral change in an infected organism. Early on, there had been a tendency to accept all behavioral changes in parasitized hosts as beneficial for the parasite, without testing alternative hypotheses. Although acknowledging the fact that numerous alterations in infected hosts were undoubtedly adaptive for parasites, two important papers [96,113] championed a more careful and rigorous approach to the study of the evolution of

TABLE 18.2. Major Groups of Metazoan Parasites of Metazoan Hosts, with Common Names (from [121])

Parasite taxon	Common name	Minimum number of living species
Phylum Mesozoa	Mesozoans	>80
Phylum Platyhelminthes[a]		
Class Cercomeridea		
Subclass Trematoda	Flukes	>20,000
Subclass Monogenea	Monogeneans	>20,000
Subclass Cestoidea	Tapeworms	>20,000
Phylum Nemertinea[a]	Ribbon worms	>10
Phylum Acanthocephala	Thorny-headed worms	>1,200
Phylum Nematomorpha	Hairworms	>350
Phylum Nematoda[a]	Roundworms	>15,000
Phylum Mollusca[a]		
Class Bivalvia[a]	Bivalves	>600
Class Gastropoda[a]	Snails	>5,000
Phylum Annelida[a]		
Class Hirudinea[a]	Leeches	>400
Class Polychaeta[a]	Polychaetes	>20
Phylum Pentastomida	Tongue worms	>100
Phylum Arthropoda[a]		
Subphylum Chelicerata[a]		
Class Arachnida[a]		
Subclass Ixodida	Ticks	>800
Subclass Acari[a]	Mites	>30,000
Subphylum Crustacea[a]		
Class Branchiura	Fish lice	>150
Class Copepoda[a]	Copepods	>4,000
Class Cirripedia[a]		
Subclass Ascothoracida	Ascothoracidans	>100
Subclass Rhizocephala	Rhizocephalans	>260
Class Malacostraca[a]		
Order Isopoda[a]	Isopods	>600
Order Amphipoda[a]	Amphipods	>250
Subphylum Uniramia[a]		
Class Insecta[a]		
Order Diptera[a]	Flies	>2,300
Order Phthiraptera (suborders Ischnocera, Amblycera and Anoplura)	Lice	>3,000
Order Siphonaptera	Fleas	>2,500

[a]Denotes taxa also containing free-living species.

parasite-induced behavioral changes. These papers set forth three questions that persist today: (1) Is a phenotypic change adaptive for a parasite or is it a nonadaptive and accidental result of infection? (2) Is the phenotypic change a host *adaptation* that reduces detrimental *fitness* consequences of infection? (3) What is the role of *phylogeny* in explaining parasite-induced phenotypic change? Behavioral changes can be the products of natural selection in a given host–parasite interaction, but they can also be inherited from an ancestor. In that case, they may or may not continue to confer a selective advantage to the parasite in the present system.

Present day researchers studying parasitic manipulation are cognizant of the problems described above. These difficulties require studies that are collaborations between parasitologists, evolutionary biologists, physiologists, neurobiologists, and biochemists in order to understand the complex process of manipulation.

Fig. 18.1. Adult specimen of *Dicrocoelium dendriticum* (photo: F. Thomas).

18.3 SELECTED EXAMPLES OF MANIPULATION

All adaptive changes in host behavior following parasitic infection are not necessarily manipulation. They can be responses of the host aimed at eliminating the parasite or compensating for its effects. Here, we will focus on changes in infected hosts thought to be cases of adaptive manipulation by the parasite. The list below is far from exhaustive.

18.3.1 Manipulation of Predator–Prey Encounters

There are numerous examples of trophically transmitted parasites that alter the behaviors of their *intermediate hosts* in ways that increase their vulnerability to predatory definitive hosts. Typically, the intermediate host becomes more conspicuous or less able to escape from predators [25,77,95]. The most popular example of trophic transmission in ecological textbooks is the trematode "brainworm" *Dicrocoelium dendriticum*, also called the small liver fluke (Fig. 18.1). This parasite reaches adulthood in large herbivorous mammals but uses ants as second intermediate hosts. The infected ant behaves normally during the day, but when temperatures drop, it ascends blades of grass instead of returning to the nest, and this altered behavior is thought to enhance transmission to grazing sheep. Among crustacean hosts, isopods harboring *cystacanths* of the parasitic worm *Plagiorhynchus cylindraceus* (acanthocephalan) are far more likely than uninfected ones to spend time in areas of relatively low humidity and on white surfaces where they are highly visible to bird predators (definitive hosts) [93,94]. In aquatic habitats, crustacean gammarids (*Gammarus pulex*) infected with avian- or fish- acanthocephalans (*Polymorphus minutus* and *Pomphorhynchus laevis*, respectively) display a range of behavioral changes (e.g., skimming and clinging behavior) that make them more likely to be eaten by aquatic birds and fishes. In contrast to the acanthocephalans, which remain in the host abdomen, the digenean trematode *Microphallus papillorobustus* encysts in the nervous system of the gammarid *Gammarus insensibilis* [56] (Fig. 18.2). Cerebral *metacercariae* of *M. papillorobustus* induce strong behavioral alterations (i.e., positive phototaxis, negative geotaxis, and an aberrant evasive behavior), making infected gammarids (commonly called "crazy" gammarids) more vulnerable to predation by aquatic birds. Trematodes and acanthocephalans are phylogenetically

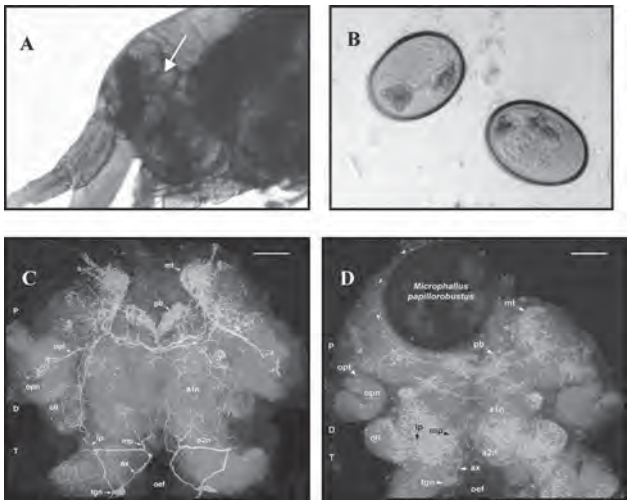


Fig. 18.2. *Gammarus insensibilis* infected by the trematode *Microphallus papillorobustus*. (A) head of a parasitized gammarids, metacercariae correspond to ovoid spheres. (B) Isolated metacercariae of *M. papillorobustus*. (C) Serotonin-containing neurons (yellow) in healthy brain. (D) Serotonin-containing neurons (yellow) in infected brain. (Montage of four stacks of 32 confocal scans showing immunoreactivity for serotonin (green label) and synapsin (red outline of neuropiles). Anterior is at the top. ax, axon of tgn; a1n, antenna 1 neuropile; a2n, antenna 2 neuropile; D, deutocerebrum; lp, lateral projections of tgn; mp, medial projections of tgn; mt, medulla terminalis; oef, oesophageal foramen; oll, olfactory lobe; opn, optic neuropile; opt, optic tract; P, protocerebrum; pb, protocerebral bridge; T, tritocerebrum; tgn, tritocerebral giant neuron. Scale bar, 100 μm.) (From [57].). See color plates.

unrelated, but they evolved under similar ecological constraints on their transmission, that is, they require the predation of the gammarids to complete their life cycle. The similarity of the behavioral changes they induce in their crustacean hosts is an evolutionary *convergence*.

Many parasites alter their hosts' abilities to escape predation. Several studies have also revealed that burrowing bivalves infected with digenean metacercariae may live closer to the surface (e.g., the cockles *Cerastoderma glaucum* and *Austrovenus stutchburyi*, respectively parasitized by *Meiogymnophallus fossarum* [9] and *Curtuteria australis* (Fig. 18.3 and 18.5) [134].

This can be interpreted as a way for the parasite to increase the probability of predation by bird final hosts such as oystercatchers. The life cycle of a different trematode, *Cainocreadium labracis,* which involves two intermediate hosts, illustrates a behavioral change resulting, as in the bivalves above, from a collective action by infective stages. The *cercariae* of the trematode crawl on the sand surface, as does the second intermediate host (generally a gobiid fish). More than 80% of the encysted metacercariae are found in muscles directly involved in gobiid swimming. Accordingly, the ability of infected *Gobius* to escape is compromised when they are attacked by the definitive host, the sea bass *Dicentrarchus labrax* [87]. Similarly, metacercariae of *Diplostomum spathaceum* decrease the visual acuity of freshwater fish by accumulating in the lens of the eye. As a result, infected fish have a reduced ability to detect predators, especially piscivorous birds (definitive hosts) [128]. Aeby [5] demonstrated that the coral-feeding butterflyfish *Chaetodon multicinctus* from Hawaiian reefs significantly prefer foraging on polyps (*Porites* sp.) that are infected by the trematode *Podocotyloides stenometra*. Infected polyps become swollen and pink (Fig. 18.4) and are easier to capture, as they are no longer able to adequately retract into their protective coral skeletons. The cestode *Ligula intestinalis* can grow to be 20 cm long and 1cm wide in the body cavity of cyprinid fishes (intermediate host) (Fig. 18.5). Infected fish not only behave differently from uninfected fish but also develop a rotund shape that is visible to birds [86]. Studies with rats

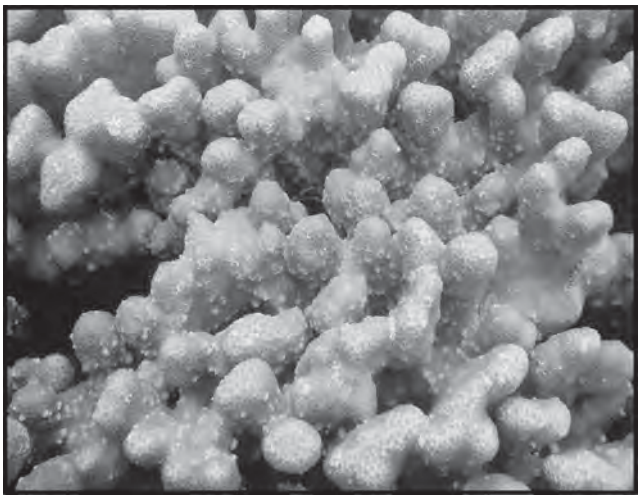
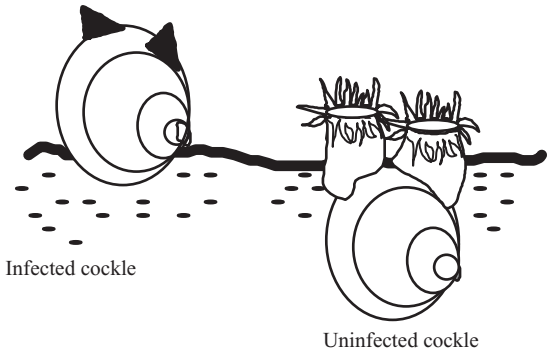


Fig. 18.4. Infection with the trematode *Podocotyloides stenometra* causes coral polyps to become swollen and pink (photo: Greta Aeby). See color plates.

(a)



(b)

Fig. 18.3. Parasitized cockle *A. stutchburyi* lying at the surface of the mud with the two most common invertebrate species living on its shell, the limpet *Notoacmea helmsi* and the anemone *Anthopleura aureoradiata* (photo: F. Thomas).

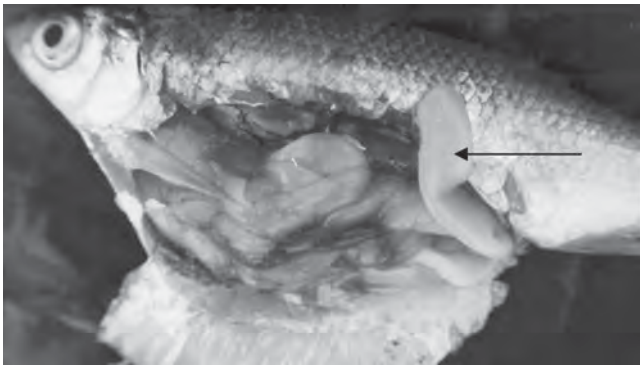


Fig. 18.5. Cyprinid fish infected by *Ligula intestinalis* (photo: Geraldine Loot).

infected with the protozoan parasite *Toxoplasma gondii* also provide compelling evidence for manipulation: *T. gondii* enhances predation of intermediate hosts by the feline definitive host by reducing the rat's innate fear of cats and their associated smells [12].

Limb *autotomy* is a way for parasites to achieve trophic transmission without killing their hosts [77]. The spiny sand crab *Blepharipoda occidentalis*, like most other crabs, has the ability to autotomise a limb to escape predation. Metacercariae disproportionately infect the crab's claws, suggesting that they might become transmitted during one of these successful escapes from an attack. Finally, bivalve mollusks from the genus *Abra* also partially autotomise their *siphons* when the latter are "full" of the metacercariae of *Paratimonia gobii*. Once detached, the siphon moves about on the bottom like *benthic* invertebrate, attracting gobies that then ingest these small parasite-stuffed morsels [87].

18.3.2 Manipulation of Habitat Choice

Parasites can also manipulate host habitat choice. Curtis [28] found that some digeneans such as *Gynaecotyla adunca* drive their molluscan first intermediate hosts toward beaches for the release of cercariae close to amphipods and crabs (second intermediate hosts). Hairworms (phylum Nematomorpha) and mermithid nematodes (phylum Nematoda) are parasitic in arthropods when juveniles, but they are free and aquatic when adults. Insects harboring mature nematomorphs seek water and jump into it, thereby allowing the parasitic worm to reach its reproductive habitat (Fig. 18.6) [146]. A similar water-seeking behavior is observed with the beach hopper amphipod *Talorchestia quoyana* parasitized by the mermithid nematode *Thaumamermis zealandica* (Fig. 18.7): the parasite induces the host to burrow more deeply than healthy amphipods and the adult worm emerges from the host into moist sand at these greater depths. Finally, several fungus species (called "enslaver" fungi) make their insect hosts (such as flies [88] or ants [85]) die perched near the top of plants in a position that facilitates the *dispersal* of *spores* by the wind.

18.3.3 Other Kinds of Manipulation

Mermithid nematodes can also feminize the morphology and behavior of male insects when parasite transmission depends on female-specific behavior [152]. Parasitic wasps can make their spider host weave a special cocoon-like structure to shelter the wasp *pupae* against heavy rain [17,39], or can even cause the host to seek refuge within curled leaves to protect pupae from *hyperparasitoids* [16]. Viruses may stimulate superparasitism behavior in solitary parasitoids and thus achieve horizontal transmission [153]. *Sporocysts* of the trematode *Leucochloridium paradoxum* develop in the snails' (*Succinea putris*) tentacles and make them look like colorful caterpillars (Fig. 18.8). These altered tentacles may attract birds (definitive hosts). The rat tapeworm *Hymenolepis diminuta* increases the life span of its intermediate host (the beetle *Tenebrio molitor*), a phenomenon that, in itself, can enhance parasite transmission [63].

18.3.4 Manipulation by Vector-Borne Parasites

Many of the most harmful parasitic diseases of humans are transmitted by blood-feeding insect *vectors*. Selection favors parasites that can manipulate their vectors to enhance transmission [48]. A common strategy used by vector-borne parasites is to increase contact between the vector and the vertebrate host(s) (reviewed in [62]). For instance, in a variety of *Leishmania*/sand fly associations (see Chapter 6), infected flies exhibit increased probing behavior due to difficulties in ingesting the blood meal. An occlusion of the stomodeal valve prevents blood from flowing into the fly midgut. Parasite-induced changes in probing behavior have also been associated with malaria-infected mosquitoes, although a different mechanism is involved [71,72]. As with infected sand flies, parasitized female mosquitoes make many attempts to feed and thus visit many different hosts, each time depositing parasites at the feeding site. Malaria *sporozoites* apparently induce a reduction in salivary apyrase activity, an enzyme that counters host hemostasis and promotes easier and longer blood feeding. At present, it is not known whether this phenomenon results from an inhibitor produced directly by the parasite or from mechanical damage created during tissue invasion. Reduced efficiency of blood meal location has also been attributed to other parasite infections in vectors such as *Rhodnius prolixus* (a blood feeding true bug, vector of Chagas disease in Latin America) infected with *Trypanosoma rangeli*, tsetse flies infected with *Trypanosoma* spp., and the rat flea *Xenopsylla cheopis* infected with the plague bacterium *Yersinia pestis*. Proximal reasons for changes in feeding behavior include physical blockage of the foregut by parasites (plague-infected fleas), obscured phagoreceptors (tsetse flies infected with trypanosomes), and reduced apyrase activity in the salivary glands (*Rhodnius prolixus* infected with *T. rangeli*) (reviewed in [62]).

In many insects, the normal process of *oogenesis* is disrupted by parasites. Fecundity reduction has been frequently reported in *Plasmodium*-infected mosquitoes. Altering vector resource management may increase available nutrient reserves, which, in turn, could enhance vector longevity and hence parasite transmission. Further experiments are however needed to confirm this hypothesis in *Plasmodium*-infected mosquitoes [62].

Finally, there are an increasing number of studies suggesting that vector-borne parasites can render their vertebrate hosts more attractive to vectors, apparently by altering odor profile. *Leishmania*-infected dogs have been shown to be more attractive to sand flies than uninfected dogs [23]. Similarly, hamsters infected with *Leishmania infantum* were shown to be more attractive to female *Lutzomyia longipalpis* [106,123] than noninfected hamsters. It has been also suggested that fever induced by some parasitic infections could increase the attraction of infected individuals to vectors.

18.3.5 Are Humans Manipulated by Parasites?

The rabies virus (genus: *Lyssavirus*) is the classic example of a pathogen that can have a profound impact on human behavior. Rabies remains a serious source of mortality in the

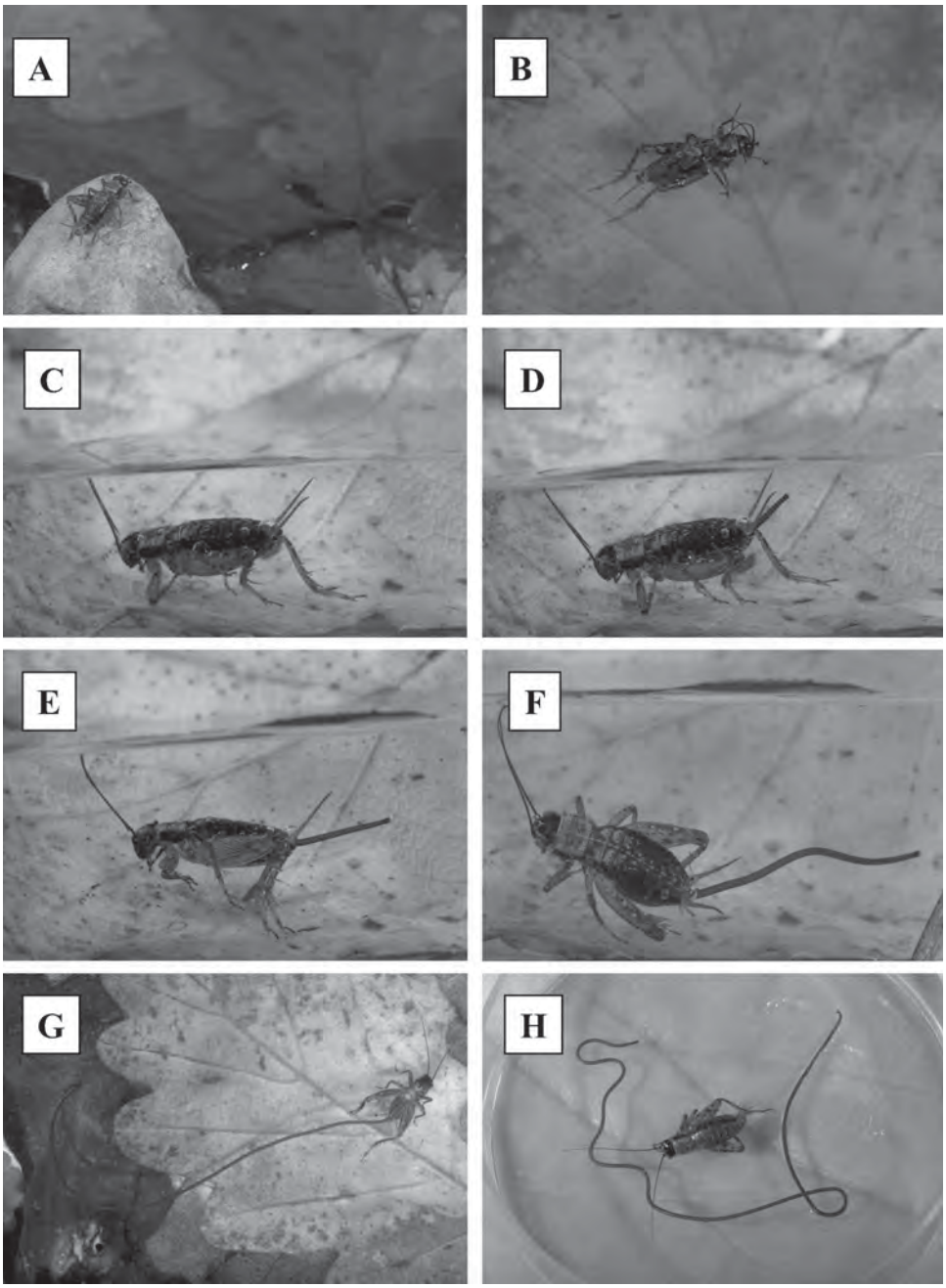


Fig. 18.6. Behavioural sequence illustrating the water seeking behaviour of *Nemobius sylvetris* followed by the emergence of the hairworm *Paragordius tricuspidatus* (photo: F. Thomas and P. Goetgheluck).

developing world, killing more people than yellow fever, dengue fever, and Japanese encephalitis do [59,127]. The rabies virus lives in the brain [127], affording the virus ample opportunity to affect host behavior. Rabid animals and humans do show changes in behavior; for example, dogs exhibiting the encephalitic (furious) form of the disease show increased aggression and biting [148]. Biting is the most effective means of transmitting rabies [127], and therefore this change in host behavior will increase parasite transmission. Rabies in humans does not provide a good example of the manipulation hypothesis, however. Humans are "dead end" hosts and do not typically transmit the disease to others [127]. Perhaps for this reason, the behavioral effects of rabies are highly variable in humans [59,127].

However, there are other examples in which a parasite-mediated change in human behavior probably does increase parasitic transmission (e.g., *Trypanosoma brucei*, which causes human African trypanosomiasis, formerly sleeping sickness). For example, Kristensson et al. ([73], Table 1, p. 246) list some of the behavioral effects of *T. brucei* and other parasites, dividing them

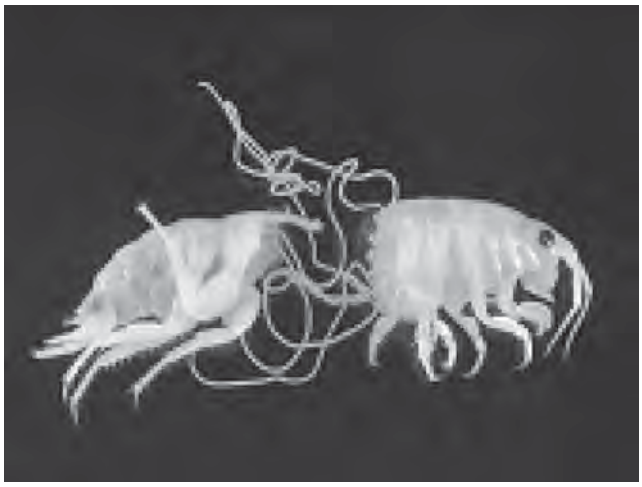


Fig. 18.7. The talidrid amphipod *Talorchestia quoyana* harboring the manipulative nematode *Thaumamermis zealandica* (photo: R. Poulin).

into potential benefits for the person and for the parasite. In some cases, the change in host behavior (e.g., increased lethargy) could benefit both. Unfortunately, most parasite-induced changes in human behavior are identical to various aspects of "sickness behavior," that is, a host response to infection [73], making it difficult to determine whether the change benefits the host or parasite (see also [51]).

Parasites that inhabit the central nervous system (CNS) are in a prime location to manipulate host behavior. In Europe and other parts of the world, one of the most common parasites of humans is the protozoan *Toxoplasma gondii*, and it lives in the nervous system. The normal intermediate hosts of *T. gondii* are rodents, but the parasite develops well in humans, too; cats serve as definitive hosts. The behavioral alterations induced by the parasite in rodents are compatible with the manipulation hypothesis, that is, they lead to an increased risk of predation by cats [12]. In humans, *Toxoplasma* infections result in slight personality changes and reduced psychomotor performance [41–45,53,75,156,157]. Because cats do not normally prey on humans, these behavioral changes are of no apparent value to the parasite. They could be manifestations of mechanisms evolved in the past to manipulate the normal rodent hosts, or they may be mere coincidental pathology shown by infected humans.

Some of the most plausible examples of the manipulation hypothesis in humans are provided by parasites that do not directly interact with the CNS (e.g., see [26]). For example, the pinworm *Enterobius vermicularis* is an intestinal nematode of humans, particularly children. Females of this parasite lay their eggs around the anus and induce an intense itching in the anal area. This behavioral manipulation is simple but very effective. The microscopic eggs of the parasite end up on fingers and are then transmitted to others. Other pathogens and parasites can induce sneezing/coughing or diarrhea (induced by most of the parasites that actively multiply in the intestine, such as *Giardia*), resulting in dissemination of infective stages to the outside environment. We must however remain cautious about assuming that these behavioral changes result in increased parasite transmission without supporting empirical evidence [26]. Finally, some other well-documented behavioral impacts of parasite infection in humans can safely be categorized as nonadaptive for the parasite, and thus be ignored in the context of the manipulation hypothesis. For instance, several intestinal nematodes (*Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms) are ubiquitous parasites in tropical and developing countries. They are also known to impair cognitive function and learning abilities in children, and productivity and wage-earning capacity in adults (e.g., [17,103,104]). These behavioral symptoms are also associated with malnutrition and anemia, and when associated with nematode infections, they are probably only the mere consequences of the parasites' impact on the host's nutritional status and general health.

Fig. 18.8. The snail *Succinea putris* parasitized by *Leucochloridium paradoxum* (photo: P. Vogel).

18.4 HOW DOES THE PRESENCE OF A PARASITE ALTER HOST BEHAVIOR?

Parasites can use both direct and indirect mechanisms to alter host behavior. Parasites can alter host behavior directly by interacting with the host's nervous system or muscle. For example, a parasite may secrete/excrete a neuroactive substance resulting in changes in host behavior. Parasites can have indirect effects on host behavior by affecting host tissues other than neurons and muscles, resulting in host-mediated changes in behavior. For example, the presence of a parasite can influence host development, intermediate metabolism and/or immunity, leading secondarily to an alteration in host behavior.

Both indirect and direct mechanisms can lead to increased transmission of parasites [1]. When researchers discuss "parasitic manipulation" of host behavior, they are usually referring

to an active process in which the parasite expends energy to produce a direct effect on the behavior of its host. However, because changes in host behavior can also be induced indirectly, parasites could alter host behavior with no additional energetic costs beyond those required to survive in the host (e.g., a suppression of host immunity that leads to a fortuitous change in host behavior). Moreover, parasitic waste products may also influence host behavior, and therefore even direct parasitic effects may be energetically cost-free for the parasite. The ecological ramifications of costly parasitic manipulation versus cost-free fortuitous changes in host behavior will be different, even if both are adaptive for the parasite (e.g., [79]). Unfortunately, differentiating between these two possibilities is problematic, as we discuss below.

18.4.1 Direct Effects

Demonstrating that secretions/excretions from a parasite act directly on host neurons has proven difficult. Part of the problem lies with the complex interactions between immunity and the nervous system. When parasites invade any tissue, including the CNS, they typically envoke complex, but poorly understood, immune cascades [73,125,129,149]. Activating these immune cascades also results in the release of neuroactive compounds [29], and these can cause a variety of changes to the brain and behavior [11]. These immune–neural interactions make it difficult to determine whether a change in host behavior is a direct effect of the parasite or a result of the host's immune response. Moreover, some parasites secrete chemicals identical to those secreted by the host's immune system [69,73]. For example, the trematode *Schistosoma mansoni* secretes β-endorphin and other opioid peptides [69]. These substances affect both immune [38] and neural function [69]. In infected hosts, both opiate and opioid levels increase in the serum and CNS, but it is unclear whether the source is the host or the parasite [69,122]. Pharmacological tests show that these compounds play a causal role in the changes in host behavior [69]. Nevertheless, *S. mansoni* has probably been selected to secrete these compounds in order to suppress local immune responses [122], not to induce changes in host behavior. Regardless of whether *S. mansoni* affects host behavior directly or indirectly, the change in behavior may be an unavoidable consequence of parasite-induced immunosuppression.

To further demonstrate the difficulty in determining the roles of parasite and host in mediating host behavioral change, we discuss here the induction of aggressive behavior in mammalian hosts by the rabies virus, already mentioned in Section 18.3. The rabies virus could increase its transmission by infecting and then manipulating areas of the brain important for regulating aggression. This hypothesis is supported by studies showing that rabies virus can alter the neuronal function of infected cells [65,74]. However, closer examination of the evidence reveals complexities in the rabies–host interaction that suggest that a direct parasitic effect on the host cannot entirely explain the increase in host aggression. Virus distribution alone cannot explain the clinical features of rabies [59]. The rabies virus preferentially localizes in the brainstem, thalamus, basal ganglia, and spinal cord [59], areas that are not directly involved in regulating aggression [109]. Therefore, it is unlikely that the virus increases aggression by directly manipulating infected neurons.

Moreover, aggression is frequently absent in infected hosts [127]. Rabies has two classic forms, the "furious" (encephalitic) and the "dumb" (paralytic) [59]. Both forms exhibit increased salivation, but only victims of encephalitic rabies exhibit increased aggression. Virus distribution in the brain is the same in both forms [59]. One difference that has been found between the two forms is that patients with encephalitic rabies tend to have intact T-cell immunity and mount a robust cellular immune response against the virus [59]. Hemachudha et al. [59] argue that it is the immune responses generated by the host that are responsible for the increased aggression seen in some rabies victims. Hemachudha et al. [59] postulate that infection of the brainstem induces production of *cytokines* by the host's immune system and that these compounds then modify the functioning of limbic system structures (brain structures involved in the control of aggression [109]). Further evidence that the increased aggression observed in some rabies victims is due to a host-generated immunopathology is that the increased aggressive behavior that occurs in humans during the final phase of rabies is also seen in other neurological disorders (both infectious and noninfectious) and is not specific to rabies [59]. This change in behavior in neurological patients is probably caused by immune-generated destruction of the CNS (e.g., inflammation [11]). In rabies, the physiological details of an individual host's immune response may play a critical role in determining whether the virus can "manipulate" its host.

In systems in which the host is an invertebrate, the mechanisms mediating host behavioral change may be easier to identify. Nevertheless, even in these systems, demonstrating that a parasite secretes a neuroactive substance, showing that the substance alters neuronal function and finding that the altered neuronal function is causally linked to the change in host behavior are difficult. Recently, Helluy and Thomas [57] suggested that the degeneration of discrete sets of serotonergic neurons in *G. insensibilis* infected by the cerebral trematode *M. papillorobustus* (see Fig. 18.2C and D, Section 18.3) might contribute to host manipulation. However, definitive evidence demonstrating the causal link between serotonin levels and the change in behavior is still lacking. In the *Gammarus lacustris–Polymorphus paradoxus* system, exogenously supplied serotonin can mimic the effect of parasitism on some host behaviors [58], and hosts show an increase in the number of varicosities exhibiting serotonin-like immunoreactivity [89]. Maynard et al. [89] included the important control of examining the CNS of gammarids infected with a different acanthocephalan, *Polymorphus marilis*, which does not induce a change in host behavior. *P. marilis* does not alter serotonergic staining in the host, demonstrating a correlation between the change in host serotonin-like immunoreactivity

and host behavioral change. Holmes and Zohar [60] do not believe that *P. paradoxus* is capable of raising host serotonergic levels sufficiently to alter host behavior. They favor the hypothesis that the parasite induces the host to increase its release of serotonin [60]. However, without biochemical tests demonstrating increased serotonin levels in parasitized individuals, and whether it is the host or parasite that is responsible for its increased secretion, we cannot determine which is paying the cost of altering host behavior.

18.4.2 Indirect Methods

When parasitic alteration of behavior has been examined in detail, the change in host behavior is usually an indirect effect of the parasite [1]. There are two possible reasons for this. First, most parasites are small, and it may be prohibitively expensive for them to secrete behaviorally effective amounts of a neuroactive compound, unless the parasite resides within the CNS. It might be more efficient to induce the host to make them. Second, in order to survive, parasites must evolve mechanisms to allow them to interact with host physiology, especially immunity. It may be a small evolutionary step to co-opt the chemical connections between these systems and the host's nervous system to induce adaptive behavioral change in the host. Immune–neural connections may be especially prone to this type of disruption because of the intimate contact between the parasite and the host's immune system [1,3]. If this is a common mechanism of parasitic manipulation, then most changes in host behavior will resemble host responses to stress or infection, making it difficult to determine whether the parasite is exerting any active effect (i.e., secreting compounds that alter host behavior). For example, the trematode *Trichobilharzia ocellata* suppresses the egg laying of its intermediate host, the snail, *Lymnaea stagnalis* using both direct and indirect methods [33]. Parasitic secretory/excretory products induce the snail's immune system to release schistosomin [32]. Schistosomin decreases the excitability of neuroendocrine cells responsible for releasing the peptide caudodorsal cell hormone (CDCH) that induces egg-laying behavior [61]. Schistosomin probably mediates a stress response in uninfected snails [34]. The parasite also exerts direct effects on gene expression in the snail's CNS [35], and some of these changes may play a role in depressing egg laying in the snail [35]. Many parasites may be like this trematode and use multiple mechanisms to alter host behavior.

18.4.3 Importance of Understanding the Physiological Basis of Host Behavioral Change

Understanding how parasites alter host behavior is important for practical as well as theoretical reasons. Because many parasitic effects on behavior exploit immune–neural connections, studying these systems will increase our insight into the molecular mechanisms underlying sickness behavior (e.g. [73]). Moreover, some parasites appear to be able to induce different behaviors in different hosts by using immune–neural connections (e.g. [59]). Further study of this phenomenon will demonstrate how different types of immune responses induce different types of behavior. Such information could lead to improved therapies for life-threatening host responses such as *cachexia*. Furthermore, infectious diseases of the CNS may underlie some common forms of mental illness [11,82,149]. Studying how parasites alter brain function may aid our understanding of these disorders. Examining how parasites alter social behavior may also tell us something about the evolution of the brain in vertebrates. In a recent review paper, Klein [70] reported several examples of pathogens affecting the proximate mechanisms that mediate the expression of social behaviors in vertebrates (aggressive, reproductive, and parental behaviors) in ways that may increase parasitic transmission. Interestingly, the effects of parasites on social behavior may be retained across several classes of vertebrates because parasites affect the phylogenetically primitive structures of the limbic system and related neurochemical systems [70]. Further research in this area should increase communication and cooperation among neuroscientists, parasitologists, and evolutionary biologists.

18.4.4 Implications about Parasitic Manipulation from Recent Mechanistic Studies

As demonstrated in the preceding sections, changes in host behavior are often a mix of direct and indirect effects of parasites on their hosts' CNS. For example, the host's immunological response to infection can be involved in changing the host's behavior in a manner that favors parasitic transmission (e.g., rabies). Studies attempting to differentiate between host responses (e.g., sickness behavior) and parasitic effects on behavior should keep this observation in mind. Even if a change in host behavior can be mimicked by activating the immune system, this change could still be adaptive for the parasite, and it could still be a direct effect of the parasite (e.g., by the parasitic secretion of cytokines). Furthermore, finding the correct immune challenge to test whether a change in host behavior could be a host response will not be easy. Immune responses can vary depending on the parasite [125], and different immune responses can elicit different types of behavior [4]. For example, parasites that infect the brain may induce specific changes in behavior due to local release of cytokines, a pattern of release that would not be reproduced by a systemic challenge.

In host–parasite systems in which the host exhibits a completely novel behavior (e.g., [146]), the causal connection between a parasitic effect and host behavioral change may be easier to establish. Activation of a unique behavior, rather than the augmentation of a host response or a decrease/increase in a normal behavior, may also be less likely to rely on exploiting a host response to infection. Unfortunately, these types of host–parasite systems are rare and have been largely ignored by physiologists.

18.4.5 New Methods in the Study of How Parasites Manipulate Their Hosts

Proteomics is the study of all the proteins produced by a cell (i.e., the proteome). Instrumental to the study of functional genomics, it incorporates protein separation methods, mass spectroscopy, and bioinformatics on a massive scale (see Chapter 22). Until now, the studies in "Parasitoproteomics" have been done using the expression of the parasite proteome during infection by a given parasite [22,80,98], the reaction of the host proteome following an invasion by a parasite species [22,97,132,155], or the injection of immune elicitors [49,154]. Because proteomics can rapidly provide a comprehensive view of the expression of entire genomes, Biron et al. [15] recently proposed that proteomics would offer an excellent tool to study the host's (and sometimes the parasite's) genomes in action during behavioral manipulation. Current studies using proteomics to identify the mechanisms of parasitic manipulation are in progress, and preliminary results reveal a bright future for such an approach.

18.5 ADAPTIVE VERSUS NONADAPTIVE CHANGES

An important debate concerns the adaptive nature of host manipulation. It is argued that phenotypic changes in infected hosts are not necessarily "true" parasitic (or host) adaptations. These changes may be "byproducts" of infection or ancestral legacies. Adaptation is a complex concept with several possible definitions (see [46] for review). For instance, the definition provided by Reeve and Sherman [124] – an adaptation is a phenotypic variant that results in the highest fitness among a specified set of variants in a given environment – refers only to contemporary effects of the trait on reproductive success. In contrast, the definition of Harvey and Pagel [52] – for a character to be regarded as an adaptation, it must be a derived character that evolved in response to a specific selective agent – explicitly requires an inference about history. Clearly, most researchers interested in manipulation adopt the second, historical definition of adaptation. Such historical definitions of adaptation (e.g. [52]) are reasonable, and even necessary in contexts such as that of comparative analyses; other applications may be more confusing. For instance, what about ancestral, inherited traits that remain advantageous to their bearers in derived groups? This persistent advantage may still exist within several groups of parasites such as acanthocephalans or trematodes (see Section 18.3.1.), where it seems likely that not only has the ability to manipulate host species been inherited from a common ancestor but also that these manipulations have continued to confer a selective advantage in the context of the transmission.

The term "byproducts" of infection refers to at least three types of phenomena. In the first case, the "side effects" of parasite infection are pathological consequences expressed in host behavior that have no adaptive value for the parasite or the host [91]. In the second case, "byproducts" are "coincidentally beneficial" for the parasite. For instance, parasitized hosts may increase their conspicuousness to predators (definitive hosts) if they forage more to satisfy increased energy requirements; this differs from straightforward manipulation [113]. Finally, a third type of change in host phenotype results in "fortuitous payoffs of other adaptations." For example, encysting in the host nervous system is one way to manipulate behavior; such a site may have initially been favored because it affords some protection from the host's immune system [96].

It is reasonable to reevaluate these "byproducts," and the rule of parsimony that has been used to defend the byproduct interpretation. In the first case, it is obviously difficult to demonstrate the absence of anything, be it benefit to parasite or host. In the case of the second and third categories, "coincidental benefits" and "fortuitous payoffs of other adaptations," it is easy to confuse the focus of historical selection (depleted energy? immunological protection?) and concomitant effects on transmission, especially when enhanced transmission itself may be favored by natural selection. In short, if parsimony produces a null hypothesis of "byproduct" as a standard by which to evaluate other interpretations, then that hypothesis must surely be testable [136,147]. Unfortunately, tests of the byproduct explanation of apparent manipulation are not a trivial undertaking. After all, altered behaviors that occur only sporadically are not terribly interesting unless the pattern of appearance/absence itself is somehow informative. On the contrary, if an altered behavior occurs routinely, it is then part of the suite of traits that are subject to natural selection in that association; it is unlikely to be an accident. This does not guarantee that the altered behavior is adaptive; after all, not all traits are adaptive. However, if pathology is linked to transmission, then it is highly likely that natural selection has not been blind to that pathology [7].

18.6 COST(S) OF MANIPULATION FOR PARASITES

If manipulation offers a selective advantage, does it also exact a cost in fitness? This possibility has been the focus of some discussion. Although suitable systems exist with which hypotheses about cost might be tested, no such tests have been conducted, and many workers seem to assume the existence of costs. The existence and amount of such costs are probably closely linked to the mechanism that underlies manipulation (see Section 18.4). In addition, in a broader evolutionary context, one must consider manipulative costs not only at a physiological level, but also at the ultimate level, in terms of fitness [116]. For instance, Thomas et al. [139] found that although amphipods (*Gammarus aequicauda*) are capable of mounting an immune response against invading parasites (involving both encapsulation and melanization), they use this cellular defense reaction only against the manipulative trematode *M. papillorobustus* and never against three other species of non-manipulative trematodes commonly

found in the abdomen. Further, encapsulation is targeted almost exclusively at *M. papillorobustus* metacercariae encysting in the host's cerebral region, that is, at those individual parasites inducing the manipulation, and not at their non-manipulative conspecifics encysting in the abdomen. In this case, manipulating the host is thus associated with higher risk of mortality for the parasite.

Assuming that manipulation is costly for parasites, then the potential for cost-sharing by manipulative parasites poses other basic questions [112]. Despite theoretical expectations of cooperation among manipulative conspecifics, very few studies have explored this issue. From data collected in the field, Brown et al. [18] found no evidence that co-occuring metacercariae of *M. papillorobustus* benefit from the presence of conspecifics in the brain of gammarids; instead, individuals in larger *infrapopulations* suffered reduced size and fecundity. The issues of cost and of adaptation versus byproduct, as well as other questions about parasite-induced behavioral changes, may have much to gain from attention to mechanisms.

18.7 MAFIA-LIKE STRATEGY OF MANIPULATION

The complexity of the interactions between hosts and parasites suggests that we may not yet fully understand host–parasite interactions. Theoretically, parasites may select for collaborative behavior in their hosts by imposing extra fitness costs on recalcitrant hosts. This interaction has been called a mafia-like strategy [130]. This process was initially proposed as a possible explanation for why several bird species accept cuckoo eggs and nestlings in their nest despite the dramatic cost to their own fitness. Cuckoos may force the bird host to tolerate non-self eggs by making the consequences of rejection more damaging than the consequences of acceptance ([159], Table 1, p. 246). In a study of the great spotted cuckoo and its magpie host, Soler et al. [130] provided empirical evidence of this phenomenon: ejector magpies suffer from considerably higher levels of nest predation by cuckoos than acceptors do, suggesting "punishment" of the ejector host when the cuckoo retaliates and destroys its clutch (see Fig. 18.9). Such retaliation favors an

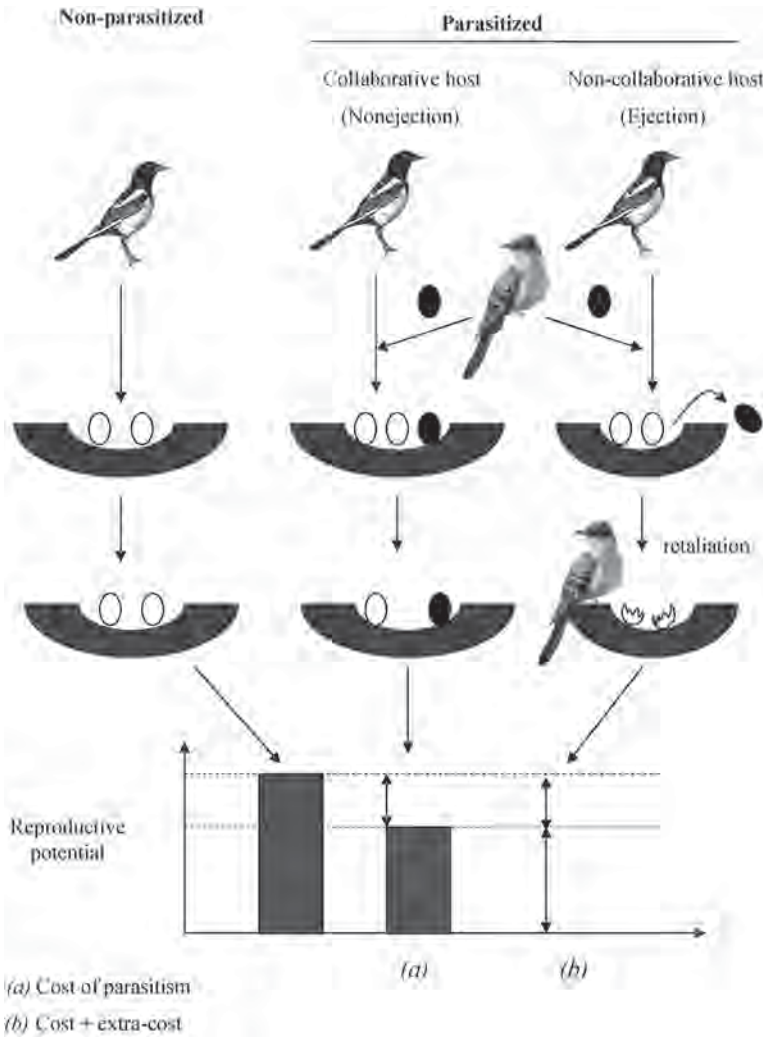


Fig. 18.9. Mafia behavior in the cuckoo *Clamator glandarius* parasitizing the magpie host *Pica pica*.

increase in "acceptor genes" relative to "rejector genes" in the host population. Such interactions could be far more common among host–parasite systems than the few existing studies might indicate.

Host species are under selective pressure not only to eliminate parasites but also to compensate for the effects of parasites when elimination is impossible. When parasites can respond to recalcitrant hosts by increasing their virulence, then host–parasite cooperation, rather than resistance, might mitigate fitness costs associated with parasitism. This scenario implies that both host and parasite can perceive a large set of fitness-related environmental cues and adjust their life history decisions (*sensu lato*) accordingly. Increasing evidence suggests that this faculty is indeed present (see [84,137,115] for parasite examples and [2,6,92,110,] for host examples). Theoretically, mafia-like strategies may evolve even when manipulative parasites strongly reduce host survival. From an evolutionary point of view, reduced survival and reduced fitness are not synonymous. Net fitness, not survival, is the primary consideration. Thus, a host that cooperates with the parasite, even to the point of "suicide" (manipulated behavior, see Section 18.3), may be better off with reduced fecundity compared to total castration inflicted by a retaliatory parasite on an uncooperative host.

Parasites affect fecundity over a wide range of outcomes, from slight reduction to total, irreversible castration. This range invites further investigations of mafia-like strategies of manipulation. For instance, noncompliance could be imposed by an investigator, and the fitness of those noncompliant hosts could then be compared to that of hosts allowed to express parasite-induced behavior. This would reveal differences between a collaborative host and a truly manipulated one, and would allow measurement of the relative costs. From an evolutionary point of view, this approach suggests that phenotypic changes in infected hosts, even when they result in clear fitness benefits for the parasite, are not necessarily an illustration of the extended phenotype of the parasite alone (*sensu* [31]). They can also be the direct product of natural selection acting on the host genome as well.

18.8 MULTIPLE PARASITES WITHIN MANIPULATED HOSTS

Another area of increasing interest is the study of the influence of manipulative parasitic species on the evolution of sympatric parasite species. Lafferty et al. [79] offer a series of ecologically based predictions about transmission strategies that should be favored by natural selection in these multispecies situations. For instance, when manipulation is costly and when both non-manipulative and manipulative parasites have a similar transmission agenda (i.e., they have the same intermediate and definitive hosts), non-manipulator parasites should increase their chance of transmission by preferentially infecting hosts that are already manipulated (hitch-hiking strategy [140,144]). In the case of two manipulative parasites that have such shared interests and produce different manipulations (e.g., color changes, behavioral changes) in ways that increase transmission additively, co-occurrence of these "co-pilots" in intermediate hosts should be favored by natural selection [79]. Finally, if an intermediate host is shared by one or more parasite species that require different definitive hosts, conflicts of interests will emerge. At least three evolutionary solutions to such a conflict have been proposed: (1) avoiding intermediate hosts containing a manipulator, (2) killing the manipulator, (3) overpowering the manipulation of the manipulator. The last instance is called "hijacking," in the case of a second trophically transmitted manipulator [79], and "sabotage" when the second parasite is not a manipulator, is not trophically transmitted, and benefits most from being in a host with a normal phenotype [138]. The number of empirical studies focusing on multiple parasites within manipulated hosts is low, but increasing (but see [20,29,36,37,40,78,107,118,117,120,140,142,144]). In order to understand these interactions, it is desirable for the entire community of parasites in manipulated hosts to be examined. In addition, a better understanding of the proximate causes of parasitic manipulation will clarify the potentially complex interactions that mediate cooperative and conflicting relationships among parasites sharing a manipulated host (see [117] for a unique example).

18.9 HOW COMPLEX ARE "PARASITICALLY MODIFIED ORGANISMS"?

A full understanding of the manipulation process requires the study of other phenotypic traits in hosts in addition to the most obviously altered behaviors. Indeed, there are several reasons to think that we have until now only studied the visible part of the iceberg; manipulated hosts may be vastly more complex than traditionally viewed.

As shown in studies on *phenotypic plasticity* and evolution, a single phenotypic change (for instance, one induced by a minor genetic mutation) can secondarily produce additional important phenotypic changes as a result of compensatory responses *via* a shift in the expression of related traits (see [102,158]). Poulin and Thomas [119] argued that the ability of infected hosts to undergo large phenotypic alterations, such as a change of microhabitat, may depend on the capacity for auxiliary traits to accommodate this novelty. To our knowledge, the idea that manipulative parasites could act as a developmental switch for several associated traits remains to be investigated.

In addition to changes resulting from plastic adjustments of the hosts to novel conditions, complex alterations of the host phenotype could result from parasites being able to manipulate several traits in their hosts. Because studies on manipulation have usually focused on the most spectacular change displayed by infected hosts, this idea has rarely been explored (but see [54,81]). When possible, manipulation of several host traits by parasites either simultaneously or in sequence should

be favored by selection. For instance, the efficiency of a behavioral manipulation could, in many situations, be enhanced by a physiological manipulation. Indeed, the display of an aberrant behavior is not only likely to be an energetically costly task for the host, but is also a period during which foraging may be reduced. Because the window of manipulation is larger for hosts with high levels of energy reserves than for those with poor reserves, natural selection should favor parasites that cause hosts to increase energy reserves. In fact, this is hypothesized to be an advantage for parasitic castrators – they are thought to shift resource allocation from reproductive to somatic (and hence, parasite) uses [114]. If intermediate hosts are "vehicles" transporting the parasites to their definitive hosts, then parasites should make sure that the "gas tank" is full. Further research is likely to reveal that parasitically modified hosts are not simply normal hosts with one aberrant trait (e.g., behavior), but instead are deeply modified organisms with a range of modifications, some of which may favor parasites, and some of which may favor hosts. Such integrative study requires collaboration among parasitologists and researchers from other disciplines, especially physiology, morphology, and developmental biology.

18.10 INTRASPECIFIC VARIATION IN MANIPULATIVE PROCESSES

Substantial variation in the intensity of the phenotypic changes is typical of many infected hosts, even when they are collected in the same environment and at the same time. As pointed out by Perrot-Minnot [108], the analysis of the intraspecific variability in these patterns is essential to understand their evolution. Two individuals may differ because they differ in *genotype*, because they differ in environmental experiences, or both. Unfortunately, the extent to which different individual parasites display different manipulative abilities and the variability in the ability of individual hosts to oppose manipulation is poorly documented. Despite the difficulty of maintaining parasites with complex life cycles in the laboratory, we need more studies aimed at identifying not only the relationship between phenotype and fitness but also the phenotypic variance and the degree to which manipulation is heritable. Such efforts will undoubtedly provide a much better basis for understanding the evolution of traits involved in the manipulative process.

18.11 MANIPULATIVE PARASITES AND ECOSYSTEM FUNCTIONING

Over the past 15–20 years, considerable progress has been made in understanding the functional importance of parasites in *ecosystems*. Much theoretical and empirical evidence has demonstrated that parasites, in spite of their small size, are biologically and ecologically important in ecosystems [24,90,99,111,131,133,135]. Little is known, however, about the more specific role(s) of manipulative parasites in these processes [79].

Parasite manipulation can first influence community structure and biodiversity in ecosystems by apparently interfering with competition between hosts. This scenario has been illustrated in salt marshes of southern France for the association between the trematode *M. papillorobustus* and the two congeneric and syntopic amphipods *G. insensibilis* and *G. aequicauda* (see also [10]). As reported in Section 18.3 (Fig. 18.2), cerebral metacercariae of *M. papillorobustus* induce strong behavioral alterations making infected gammarids more vulnerable to predation by aquatic birds. In *G. insensibilis*, metacercariae always alter behavior, as they are always cerebral [56]. Conversely, in *G. aequicauda*, metacercariae can also be abdominal; in this case, they have no particular effect on the host behavior [55]. In the field, two distinct infection patterns are observed in the two amphipod species (Fig. 18.10) [145], indicating that the manipulation exerted by *M. papillorobustus* probably acts as an important mechanism regulating the density of *G. insensibilis* populations versus that of *G. aequicauda* (see [126]). Because the higher reproductive success of *G. insensibilis* [66] is offset by its lower tolerance to *M. papillorobustus*, the *sympatric* coexistence of the two amphipod species is likely to be mediated by this manipulative parasite [145].

A second process through which manipulative parasites could influence community structure in ecosystems is through their influence on the predator community. As seen in Section 18.3, many trophically transmitted parasites adaptively change the phenotype of their hosts in a way that increases their probability of being captured by definitive hosts [25,26,77]. Predators sometimes risk infection when feeding on manipulated prey, but they also often benefit from enhanced prey capture [64,76,93,105]. In addition, most manipulative parasites in intermediate hosts apparently cause little harm to definitive hosts [76,77], so we can safely assume that predators not only have no *a priori* reason to avoid manipulated prey, they should even prefer foraging on those prey (see [5,76,93]). By increasing accessibility of prey that is normally difficult to capture, the net effect of manipulative parasites in ecosystems may be the enhancement of the trophic potential of these habitats. Unfortunately, this idea remains to be tested; there are no reports of possible positive relationships among the local abundance of manipulative parasites, food accessibility for predators and their local *richness/diversity*.

A third important mechanism by which manipulative parasites may influence processes of community ecology is through their interference with engineering processes. Ecosystem engineers are organisms that directly or indirectly modulate the availability of resources to other species by causing physical state changes in biotic or abiotic materials [67,68]. Manipulative parasites, by altering the phenotypes of their host, can either have impacts on existing ecosystem engineers or act as engineers themselves [141]. The idea that parasites could create new resources for other species by shifting

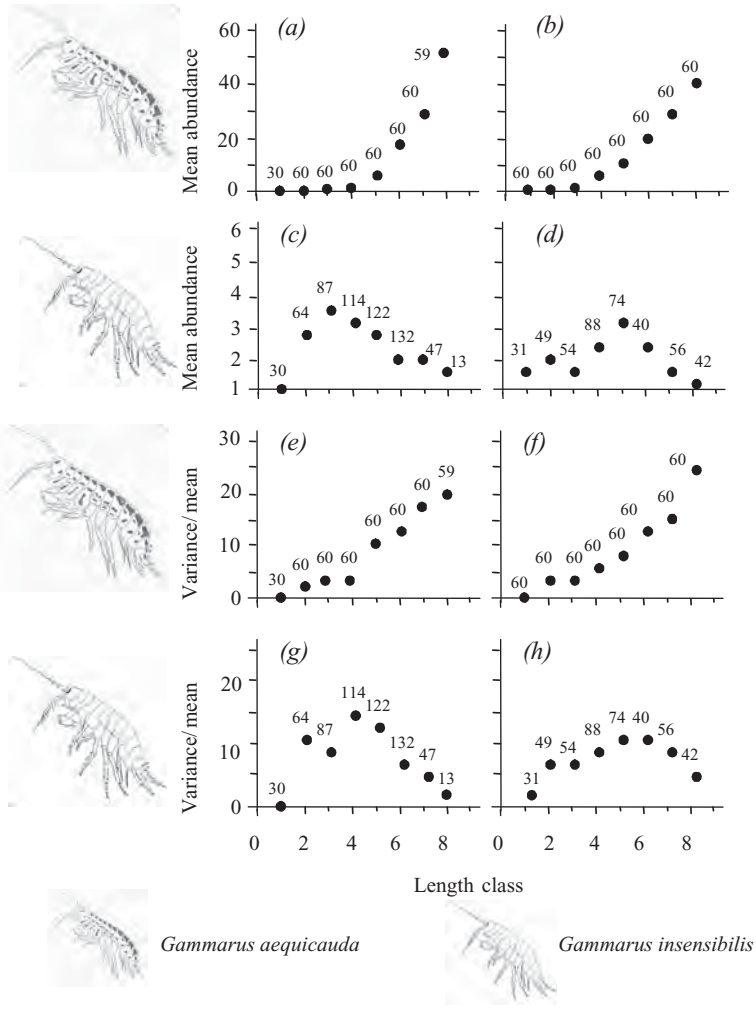


Fig. 18.10. Infection patterns (mean abundance and variance to mean ratio) of the trematode *Microphallus papillorobustus* in relation to host size in *Gammarus aequicauda* (a,b,e,f) and *G. insensibilis* (c,d,g,h). The number of hosts analyzed in each length class is indicated above each dot. Left-hand column presents data for males, the right-hand column for females (based on [145]).

the phenotype of the host from one state to another is well illustrated by the association between the cockle *A. stutchburyi*, the trematode *C. australis* and various epibiotic invertebrates ([143], see also Section 18.3, Fig. 18.3B).

A. stutchburyi lives just under the surface of the mud in many sheltered shores of New Zealand. This abundant mollusk can be considered an autogenic engineer, as its shell is the only hard substrate where invertebrates such as sea anemones (*Anthopleura aureoradiata*) and limpets (*Notoacmae helmsi*) can attach (Fig. 18.3). This cockle is also the second intermediate host of the trematode *C. australis*, a manipulative parasite that enhances its transmission to oystercatchers by altering the burrowing behavior of cockles (infected cockles remain at the surface of the mud) [134]. Manipulated cockles apparently provide a new kind of substrate for the fouling community of invertebrates. Indeed, limpets, which are normally outcompeted for space on burrowed cockles by sea anemones, significantly prefer surface cockles [143] (Fig. 18.3B). Conversely, manipulated cockles support fewer anemones than burrowed cockles do because of their greater exposure to desiccation at low tide. The manipulation exerted by the trematode *C. australis* clearly turns living material (the cockle) from one physical state (buried) into a second physical state (surface), and this act of engineering modifies both the availability and the quality of habitats for invertebrates. It seems likely that the net effect of this manipulation on the local biodiversity is positive; by reducing competition for space between invertebrates, the local coexistence of limpets and anemones is likely to be facilitated. The effect of the trematode *C. australis* extends beyond the fouling invertebrates living on the cockle shell. The presence of numerous manipulated cockles lying on the sediment modifies seabed hydrodynamics, and the reduced burrowing of infected cockles decreases bioturbation of the sediments, with the net impact being that sediment characteristics in areas of high parasite abundance differ from those in adjacent areas of low

parasite abundance. In a field experiment, the species richness and the overall density of benthic invertebrates (polychaetes, crustaceans, etc.) settling in plots with high parasite abundance were higher than in control plots with low parasite abundance [101]. Thus, a single manipulative parasite can have measurable effects on biodiversity of an entire intertidal ecosystem.

The parasite community (the trematodes *M. papillorobustus*, *Maritrema subdolum*, and the nematode *Gammarinema gammari*) harbored by the brackish gammarid *G. insensibilis* provides an interesting system for exploring how parasite manipulation can have both positive and negative effects on species richness. As seen above, the trematode *M. papillorobustus* is a manipulative parasite that increases the vulnerability of gammarids to predation by aquatic birds (definitive hosts of the parasite). Using the terminology of Jones et al. [67], this manipulation can be said to turn gammarids from a phenotype A (normal behavior) to a phenotype B (altered behavior). Thomas et al. [140] showed that the trematode *Maritrema subdolum*, which also completes its life cycle in an aquatic bird but does not alter the behavior of the intermediate host, preferentially infects phenotype B gammarids [140]. The manipulator and the "hitch-hiker" *M. subdolum* therefore seem to share interests, but the situation is quite different with the nematode *G. gammari*. Indeed, because this parasite uses the amphipod as a habitat and source of nutrition but not as an intermediate host, there is a clear conflict of interest between the nematode and the trematode. In accordance with theoretical expectations, the manipulator and the nematode are negatively associated in the field, suggesting that *G. gammari* prefer phenotype A gammarids [138]. Finally, laboratory experiments suggest that the nematode is able to "sabotage" the manipulation exerted by *M. papillorobustus*, reversing gammarids from a phenotype B to a phenotype A [138].

Many studies of parasitic manipulation have been performed without considering the ecological context in which they occur. This is unfortunate, for it compromises both our understanding of the evolution of parasitic manipulation and our understanding of the ecological consequences of manipulation within ecosystems. A full understanding of the evolution of parasitic manipulation requires knowledge of the selective pressures experienced by both the host and the parasite. Conditions used in laboratory studies as well as in semi-natural experiments may be poor approximations of processes that occur in the field. For instance, the proportion of manipulated hosts/uninfected hosts often used in predation experiments is huge compared to that frequently observed in natural conditions. How this affects the behavior of predators, and hence the conclusions derived from these studies, is not known. Most experiments do not take into account the fact that, in natural conditions, other predators unsuitable as hosts may also take advantage of the manipulation (see for instance [100]), or that several suitable host species may vary in their predation efficiency. These phenomena are nonetheless critical to our understanding of the costs and the benefits of parasitic manipulation. In some cases, certain features of parasite-induced behavioral changes seem more relevant to limiting the risk of predation by the wrong (non-host) predator than to increasing transmission to appropriate hosts (e.g. [83]). Such altered behavior in intermediate hosts cannot be understood outside its ecological context.

Finally, an important limitation of virtually all recent theoretical and experimental studies of the evolution of parasitic manipulation is the fact that these phenomena occur in a metapopulational context (e.g. [150]). Like most animal species, host and parasite species are likely to exhibit a classical *metapopulation* structure over their entire geographic range, occupying habitats that are fragmented and heterogeneous in space and/or time. In heterogeneous environments, local populations might be permanently maladapted because of migration from other habitats with contrasting selection pressures (e.g., sink populations, see [50]). Such concepts have not been tested in the context of manipulative changes, but research in this direction could well provide examples of adaptive changes that are locally maladapted. For instance, given that predator communities frequently vary in space and/or time, the fitness benefits for trophically transmitted parasites that result from manipulation differ from one place to another, with some sink populations being net importers of individuals and genes. In similar fashion, adaptive baseline behaviors of uninfected animals may vary across host ranges, thus changing the behavioral substrate on which the manipulative parasite may work [95]. General conclusions about the possible adaptive value of host changes induced by parasites must therefore be considered with caution when derived from local and/or short-term field studies. Consideration of the spatial structure of both host and parasite populations as well as the heterogeneity of environmental conditions is as desirable as it is daunting.

18.12 CONCLUDING REMARKS

The path to understanding the evolution of manipulation is a long and winding road [21]. The trait of interest is the product of the interaction between the genotypes of two different organisms, the host and the parasite. The host–parasite interactions that form the core of this research reflect evolutionary processes that have been ongoing, often for more years than we can measure. The interdisciplinary scientific interactions required to understand them have just begun.

GLOSSARY

Adaptation: A trait that evolved because it improved reproductive performance.

Autotomy: The ability of certain lower animals, such as lizards and starfish, to cast off injured body parts, such as the tail and, usually, to regenerate new ones.

Benthic: Organisms that live at the bottom of a river, lake or ocean.

Byproduct: Something that is made in the process of making something else.

Cachexia: A clinical condition in which there is a depletion of blood proteins, and fat deposits. Tissue proteins are eventually catabolized with the ultimate possibility of severe tissue atrophy and muscle wasting. There is a progressive weight loss.

Cercaria: An immature digenean, usually free swimming, produced by a sporocyst or a redia.

Congeneric: Species of the same genus.

Convergence: Two species resemble each other not because they shared common ancestors but because evolution has adapted them to similar ecological conditions.

Cystacanth: Juvenile stage of acanthocephalans that is infective to the definitive host.

Cytokines: Cytokines are chemical messengers (proteins) made by cells that influence that influence the behavior of other cell types. Cytokines produced by lymphocytes are also referred to as lymphokines or interleukins.

Dispersal: Movement of living organisms away from their previous home range. Often refers to the movement of an organism away from the home range where it was born when it matures.

Diversity: An ecological concept that incorporates both the number of species in a particular sampling area and the evenness with which individuals are distributed among the various species.

Ecosystem: A community of organisms and its environment.

Hyperparasitoid: A parasitoid that uses another parasitoid as a host.

Fitness: For a start, relative lifetime reproductive success, which includes the probability of surviving to reproduce. In certain situations, other measures are more appropriate. The most important modifications to this definition include the inclusion in the definition of the effects of age-specific reproduction, and of fluctuations of density dependence.

Genotype: The genetic constitution of an individual.

Horizontal transmission: The transmission of a virus, parasite, or other pathogens from one individual or one cell to another within the same generation, as opposed to vertical transmission through the germ line.

Infrapopulation: All of the individuals of a single species within a single host at a particular time.

Intermediate host: That host in a parasite's life cycle required by the juvenile parasite to complete its life cycle and in which some morphological change or development occurs.

Metacercaria: A developmental stage of digeneans between a cercaria and an adult; usually sequestered within a cyst in a second intermediate host.

Metapopulation: A set of partially isolated populations belonging to the same species. The populations are able to exchange individuals and recolonize sites in which the species has recently become extinct.

Natural selection: A non-zero correlation of trait variation with variation in reproductive success. The process in nature by which, according to Darwin's theory of evolution, only the organisms best adapted to their environment tend to survive and transmit their genetic characters in increasing numbers to succeeding generations, whereas those less adapted tend to be eliminated.

Oogenesis: The formation and growth of the egg or ovum in an animal ovary.

Phenotype: Observable characteristics of an organism produced by the interaction of the organism's genotype and its environment. For example, hair type, eye color, height.

Phenotypic plasticity: Sensitivity of the phenotype to differences in the environment.

Phylogeny: The history of a group of taxa described as an evolutionary tree with a common ancestor as the base and descendent taxa as branch tips.

Pupa: The last immature stage in an insect's life, just before the adult stage.

Richness: The number of species in a biological community.

Siphon: Opening in molluscs or in urochordates which draws water into the body cavity. In many molluscs, the siphon may be used to expel water forcibly, providing a means of propulsion.

Spore: Reproductive body of fungi and other lower plants, containing one or more cells.

Sporocyst: An intramolluscan, asexual developmental stage of digeneans.

Sporozoite: The motile, infective stage often present within a cyst or shell produced during sporogony.

Sympatry: Occurring in the same geographic area.

Vector: A micropredator that transmits a parasite from host to the next. Development in the vector may, or may not, occur.

Virulence: The degree of pathogenicity of a microorganism as indicated by the severity of the disease produced and its ability to invade the tissues of a host. By extension, the competence of any infectious agent to produce pathologic effects.

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CHAPTER 19

Human Genetic Diversity and the Spread of Infectious Diseases

M. Tibayrenc

19.1 INTRODUCTION: KEY CONCEPTS

The present chapter and its companion chapter 20 overlap on a number of points, emphasizing the concept that studying the impact of human and pathogen genetic diversity on the transmission and severity of infectious diseases should be done jointly, in a common integrated approach [40].

This chapter does not aim at being a comprehensive review of the present state of the art on human genetic susceptibility to infectious diseases. It is constructed around a few specific *key concepts and working hypotheses*:

- Even when examining specific parameters (here infectious diseases), it is misleading to focus only on those genes that are assumed to be more directly linked with a particular parameter. It is preferable to have a clear idea of the overall genetic diversity of our species and of the evolutionary origin of this diversity. Such a strategy has been successfully applied to the study of pathogens (see Chapter 20). Working within a comprehensive population genetics framework of the whole species in its entire ecogeographical range makes the research and study of relevant genes easier (pathogenicity, resistance to drugs).
- Genetic susceptibility to infectious diseases generally involves multigene mechanisms rather than simple monogenic backgrounds.
- Even if we knew the whole sequence of all the genes involved, this does not mean that we would understand the way they drive susceptibility to infectious diseases. Indeed, interaction between the many genes involved and between these genes and environmental factors could be very complex. Genetic susceptibility to infectious diseases is a phenotype, and only a specific case of the general fact that we know very little about how genotypes lead to phenotypes (see below).
- The overall functioning of the human genome has not yet been sufficiently grasped. This is currently true for any species. Indeed, in the present state of the art, even knowing the entire sequence of a genome says little about the way this genome works to generate specific phenotypes. To illustrate this, the whole sequence of the human genome has been now completed [12], and the sequence of the chimpanzee genome is almost entirely known [37]. However, the results of this huge effort are disappointing because the answer to why humans and chimps are phenotypically so different, although their sequence is so similar remains a total mystery. Only 1.23% of base pairs are different between the two genomes. This includes all types of sequences, including coding sequences. The implications of this amazing result are clear: considerable and heritable phenotypic differences can be compatible with very weak sequence differences. This could be true for susceptibility to infectious diseases and to the different clinical forms of a disease.
- Lastly, when human genetic susceptibility to infectious diseases is considered, the population level is as important or more important to consider than the individual level. Natural selection acts on populations as well as or more than on individuals. Geographic and climatic zones play a considerable role, because they drive pathogen diversity and distribution of pathogen species. This effect should be exacerbated in the case of vector-borne diseases, as geography and climate also have a strong impact on vector distribution.

Such concepts therefore lead to drastically different approaches than the classical approach relying on the search for a few genes at the individual level.

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Fig. 19.1. Human and chimpanzee. Although pertaining to two different species and having many phenotypic differences, they are separated by small gene sequence differences only.

19.2 EXPLORING THE GENETIC BACKGROUND OF HUMAN GENETIC SUSCEPTIBILITY TO INFECTIOUS DISEASES

19.2.1 Methodology

The basic working hypothesis underlying this research is that there are differences among individuals in terms of susceptibility to infectious diseases, and that these differences have a genetic base. This hypothesis is supported by analysis of the "sibling risk" (λ_s) or the statistical risk for the sibling to exhibit a given pathology when the other sibling does. In the case of infectious diseases, λ_s is not negligible, although lower than in the case of autoimmune diseases such as diabetes type I [11]. This is evidence for a genetic component of susceptibility to infectious diseases.

Progress in genetics has provided us with efficient tools to look for the genes involved in the susceptibility to infectious diseases. Two main approaches can be distinguished, namely candidate genes and linkage studies.

In the *candidate gene approach*, the working hypothesis postulates that a given gene or a given set of genes is involved in the susceptibility to a given disease. For example, a given gene or family of genes is suspected because it is associated with biological processes that are usually involved in infectious processes, such as antigen genes or HLA genes. Or the equivalent gene has been observed to play an important role in infectious processes in animal models. However, this last deduction could prove to be untrue. Indeed, comparable gene sequences sometimes encode drastically different functions in different animal species.

When the *linkage approach* is considered, a specific region of the genome is identified through whole genome mapping with a broad set of microsatellite markers and association analyses relying on twin/sibpair/family/pedigree studies. Thanks to powerful new technologies, systematic screening is becoming easier and cheaper, and the number of microsatellite markers available is now considerable. The null hypothesis is a total lack of linkage disequilibrium, which is evidence for free recombination between the marker used and the pathological trait explored. If the recombination rate is significantly lower than 0.5, the linkage hypothesis is retained. This suggests that the region identified by the microsatellite mapping harbors genes that have something to do with the pathology considered. To look for this type of association, nonparametric tests are not dependent on a working hypothesis on genetic inheritance. However, they are less powerful than parametric tests. The more widely used parametric linkage test is the lod (logarithm of the odds) score [29]:

$$\text{Lod} = \log 10 \left[\frac{\begin{array}{l}\text{(probability of data if disease and}\\ \text{marker are linked)}\end{array}}{\begin{array}{l}\text{(probability of data if disease and}\\ \text{marker recombine freely)}\end{array}} \right]$$

19.2.2 A Limited Harvest

A genetic component for susceptibility to infectious diseases has been postulated for many different diseases, including HIV [14], tuberculosis [11], hepatitis C [4], leprosy [2], schistosomiasis [15], visceral leishmaniasis [7], and malaria [16]. However, the links evidenced, although clear, are generally not that strong, and in many cases, the search for the responsible genes has been disappointing.

19.2.3 Problems Encountered

The gene identification methods exposed above are more successful when the following conditions are brought together: (i) when the pathological trait (phenotype) is clearly and precisely defined, which is the case, for example, for the different clinical forms of leprosy; (ii) when the genetic component of susceptibility is strong; (iii) when a limited number of genes are involved; and (iv) when precise hypotheses on the Mendelian inheritance of the involved genes are available. Needless to say that such favorable situations are probably the exception rather than the rule when genetic susceptibility to infectious diseases is considered [11]. This may be why the search for susceptibility genes has been somewhat disappointing. It is probable that the genetic control of infectious diseases depends on a complex interaction between (i) many genes and (ii) these genes and environmental parameters. Also, it is probably misleading to limit oneself to an approach exclusively based on individuals. *Natural selection targets more the population than it does the individual*. With infectious diseases, interaction phenomena among members of a given group play a major role. The term "herd immunity" [5] designates a group's overall level of immune defense. Let us consider the case where almost all members of a human population are vaccinated. The few individuals who are not vaccinated are protected by the others because epidemics cannot spread within the group. Such group phenomena most probably play a major role in the evolution of genetic susceptibility to infectious diseases and are not sufficiently considered by the classical research of susceptibility genes. This is why it is so important to consider the population/ethnic level of analysis, because obviously different populations and ethnic groups have been subjected to different pathogens and different selective pressures.

19.3 HUMAN DIVERSITY REVEALED BY NEUTRAL/HISTORICAL GENETIC MARKERS

As noted above, rather than analyzing genes of susceptibility (or supposedly so) alone, it is wiser to build a general population genetics framework of the species considered first [40]. Neutral genes and neutral polymorphisms are the best choice because they are considered to be passive markers of the time elapsed. In other words, differences in neutral genes between two populations or species are considered to be proportional to the time elapsed since these two populations or species shared common ancestors ("historical markers").

By definition, a neutral polymorphism is not influenced by natural selection. Although it is extremely difficult to strictly verify this assertion, many characters are considered

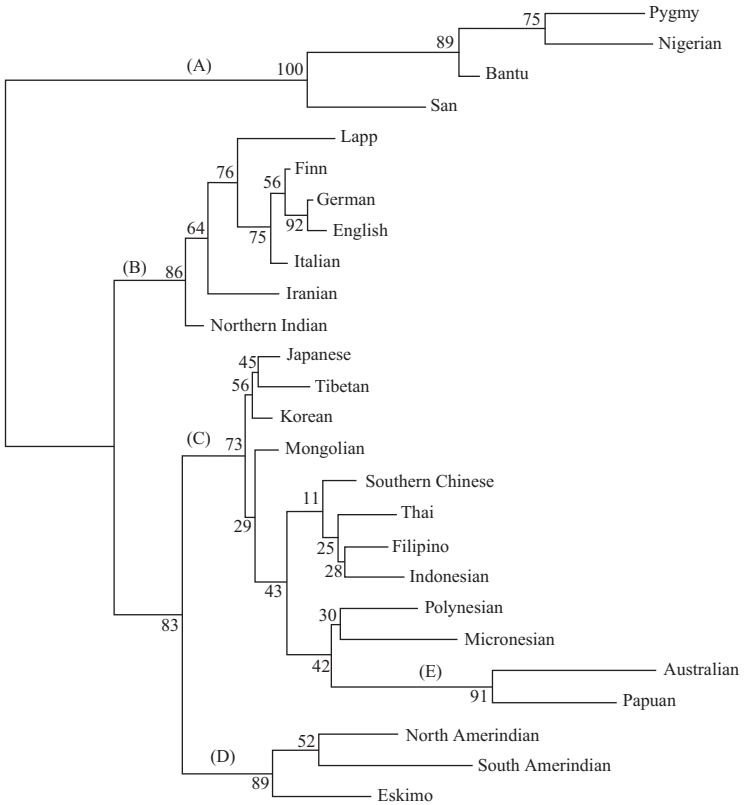

Fig. 19.2. A phylogenetic tree of the main human ethnic groups designed from isoenzyme markers (after [30]). Subdivisions match the ethnic groups classically distinguished by physical anthropology [42] and are supported by strong bootstrap values, although they do not correspond to phylogenetic subdivisions

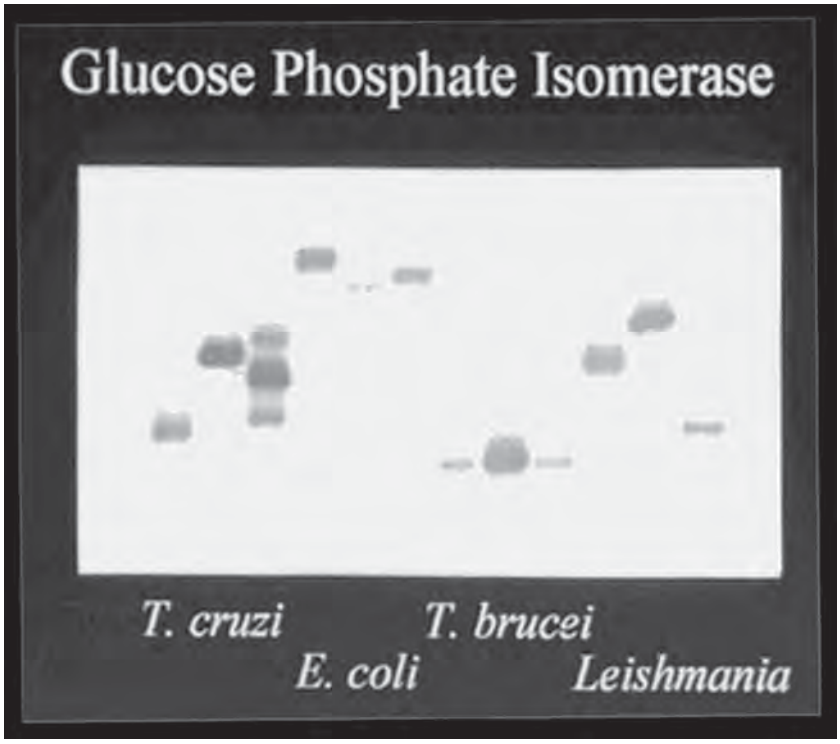

Fig. 19.3. An isoenzyme gel for the enzyme glucose phosphate isomerase deciphering the genetic variability of several kinds of pathogen (see Chapter 20). From left to right: *Trypanosoma cruzi*, the protozoan parasite responsible for Chagas disease in Latin America; *Escherichia coli*, a bacterium; *Trypanosoma brucei*, the agent of human African trypanosomiasis, formerly known as sleeping sickness; *Leishmania* spp., protozoan parasites responsible for leishmanioses. Only one technique is able to analyze different kinds of organisms ("generalist marker" [39]). Different isoenzyme bandings reveal genetic differences among and within species.

neutral. For example, in the genetic code, "synonymous mutations" have no consequences on the amino acid encoded. Noncoding sequences (introns, spacer DNA sequences) are considered neutral.

Two parameters could lower the correlation between how long two populations have been separated and the genetic distances by which they differ: (i) when the populations are not strictly separated and exchange migrants (the case for human populations), the genetic distances between them are underestimated, as migrants tend to homogenize the gene pools of the populations compared; and (ii) when founder effects interfere, which increases genetic distances. Absolute datings ("these populations split apart 123,351 years ago on a Friday morning") should therefore be considered with caution, which is not always the case. Relative datings are more reliable. In Figure 19.2, it is can be safely inferred that Iranians and Europeans shared more recent common ancestors than Africans and Chinese.

By nature, neutral polymorphism has a limited predictive power on adaptive polymorphism (see also Section 19.4). On the other hand, it gives a general population genetic framework of the species under study, shows the degree of genetic similarity between populations, the rates of migration and genetic exchange, and provides many other relevant parameters. As noted earlier, it is therefore convenient to have this general picture drawn before studying specific genes (for example, in the human species, those genes that are specifically involved in the transmission and severity of infectious diseases). A specific marker, isoenzymes, has played a major role in this kind of study, both for human and for pathogen (see Chapter 20) population genetics.

19.3.1 The Isoenzyme Revolution

In the late 1970s, a revolution occurred in population genetics. Until then, the theoretical base of this discipline had remained very speculative because a direct analysis of the genetic polymorphism was not available. The isoenzyme technique provided a direct measure of genetic polymorphism for the first time. Admittedly, this polymorphism dealt with a special category of genes, those that code for enzymes, the biochemical catalyzers able to lower the level of energy necessary for all metabolic reactions in the organism. However, provided that a sufficient number of enzyme loci were considered (multilocus enzyme electrophoresis or MLEE), it was thought that the polymorphism evidenced was representative of the entire genetic variability of the

organism. Isoenzymes were very fashionable in the 1970s and 1980s. An incredible number of papers dealing with nearly the entire living reign were published. Indeed, MLEE is the perfect example of a "generalist" genetic marker, that is, a marker usable for any kind of organism (Fig. 19.3) [39]. This wide use of isoenzymes for a number of species, including for those organisms that can be crossed in the laboratory such as fruit flies, makes the Mendelian inheritance of this polymorphism perfectly known. It is considered neutral and therefore is a time marker.

MLEE has been widely applied to the analysis of human genetic variability [30].

19.3.2 A Now Classical Pattern

MLEE made it possible to gather the main results that have been confirmed and refined by more modern technologies such as microsatellites, Alu insertion sequences, or single nucleotide polymorphisms (SNPs): (i) with historical markers, about 85% of the diversity is found within populations (e.g., Europeans) and only 15% is due to differences between populations (e.g., between Europeans and Africans). Differences between populations can be measured by the F_{st} statistics (Box 19.1). (ii) However, when phylogenetic trees are designed with MLEE, they recover the ethnic subdivisions classically used in physical anthropology quite well (Fig. 19.2). It is interesting to note that the main subdivisions seen on this tree are supported by high bootstrap values. This results from the presence of certain alleles that have much higher frequencies in some ethnic groups than in others. This property has been widely used with more modern techniques to elaborate sets of markers able to identify ethnic groups (ancestry informative markers or AIMs) [31]. The company DNAprint Genomics (http://www.dnaprint.com/welcome/home/index.php) markets kits that can identify the ancestry of any individual and even his or her degree of admixture with a high rate of reliability. AIMs have been used in forensic medicine and are also applied to the compared genetic susceptibility of different ethnic groups to given pathological traits (see below).

The high bootstrap values observed in the tree in Figure 19.2 require cautious interpretation of the results of this statistics. Many scientists consider that high bootstrap indices are a strong indication of the robustness of phylogenetic lines. The subdivisions shown in Figures 19.2 and 19.4 are by no means phylogenetic lines but only geographical populations of the same species that have been isolated by geographical distance for a relatively short time: it is considered that all present human populations shared common ancestors about 100,000 years ago [36].

It is remarkable that analyses based on a wide range of modern molecular markers have fully confirmed the results obtained by the pioneering MLEE studies of the 1970s (see Fig. 19.2), by (i) confirming that most of the genetic diversity recovered is within rather than between populations and (ii) clearly identifying the ethnic groups described by classical anthropology [42] (Fig. 19.4).

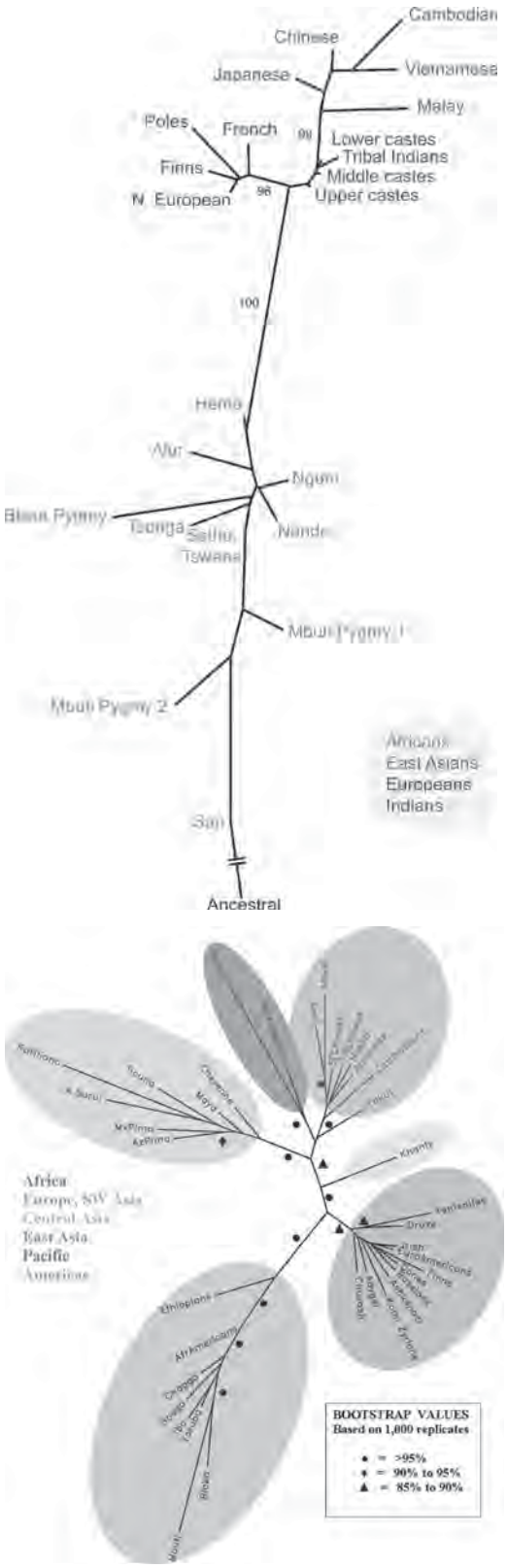

Fig. 19.4. Phylogenetic trees based on modern molecular markers. The overall pattern is comparable to that of Figure 19.1, clearly distinguishing between classical ethnic groups, although the overall difference between groups is limited (after top [22] and bottom [41]). See color plates.

(a)

(b)

(c)

Fig. 19.5. Hereditary transmission through three generations of nose shape in the Bourbon dynasty of kings of France. (a), (b) and (c) Louis XIV, Louis XV, Louis XVI. See color plates.

BOX 19.1: *F*-STATISTICS

A method proposed by Wright [43] for describing the genetic population structure with three *F*-statistics, F_{is}, F_{it}, and F_{st}, whose relationships are quoted as follows:

$$(1-F_{it}) = (1-F_{st})\,(1-F_{is})$$

F_{is} can be defined as the correlation between homologous alleles between individuals in the local population and F_{it} as the allelic correlation in the total population. They are also called fixation indices (F_i) and describe departures from Hardy–Weinberg expectations within local populations versus the total population:

$$F_i = (h_0/h_e)$$

With h_0 = observed heterozygosity and h_e = expected heterozygosity. $F_i = 0$ corresponds to Hardy–Weinberg equilibrium; If $F_i > 0$, there is a deficit of heterozygotes, due to either inbreeding ($F_{is} > 0$) or inbreeding + population subdivision ($F_{it} > 0$).

The F_{st} component is a commonly used measure of *population subdivision* (if $F_{st} = 0$, $F_{it} = F_{is}$) and can be interpreted as the variance of allelic frequencies among populations.

19.4 GENETICALLY DRIVEN PHENOTYPIC DIVERSITY

19.4.1 The Abyss Between Genotype and Phenotype

It is hard to believe for nonspecialists, but our knowledge in genetics at the dawn of the twentieth century is both immense and in its infancy. People who look at the photograph of the editor-in-chief of this book (see biographical sketches) will note that he does not have a thick head of hair. Nobody on earth, even the finest geneticist, knows the genetic background of balding. This is true for most phenotypic traits that give rise to the appearance of human beings (and other living creatures): the shape of the nose, the eyes, the color of the skin, size, and so on. We have only hypotheses, we know that balding has something to do with gender, as women are rarely affected by this disgrace. Testosterone obviously plays a role, but many very masculine men do not become bald, and the contrary is true for many less masculine men. To make a long story short, we do not know the first word of the genetic mechanisms of balding. However, this character has an obvious familial component, which suggests that it is genetically driven.

19.4.2 Phenotypic Traits that have an Obvious Genetic Basis

Many morphological traits have strong heritability, even if they are also influenced by environmental parameters. This is the case for size, body shape, skin color, hair texture, and so on. Everybody has been struck by the facial resemblance among members of the same family, which can jump over generations. This is illustrated by the hereditary character of face morphology, especially the shape of the nose in the Bourbon dynasty of kings of France (Fig. 19.5).

When phenotype is taken in a broad sense (any observable character apart from the genome itself), this remains true. Most phenotypic traits have a strong heritability, and this seems to be the case for susceptibility to infectious diseases (see Section 19.2 above).

It is interesting to note that genetically driven phenotypic variability among populations can be much higher than the genetic differences among them as measured by historical markers. When the F_{st} component (see Box 19.1) of these historical markers is as low as 0.15, the same component measured for skin color among human populations ranges from 0.6 to 0.9 [20]. It is true also for face morphology: the degree of variability between different human populations is far higher than the same parameter for two different ape species, chimpanzee, and bonobo [35]. From these facts, it can be said that genetically driven phenotypic differences among populations and ethnic groups are more extreme than the genetic differences evidenced by historical markers (Figs. 19.2 and 19.4), although they roughly follow the same pattern. The working hypothesis that comes to mind is that genetic diversity related to susceptibility to infectious diseases also follows this pattern to some extent. In other words, this parameter explored in the present chapter has something significant to do with populations and ethnic subdivisions.

19.5 ARE RACES AND ETHNIC GROUPS BIOLOGICALLY MEANINGFUL AND MEDICALLY RELEVANT?

19.5.1 The Biological Nature of Ethnic Groups/Races

The biological relevance of races and ethnic groups is a highly ideologically connoted debate. As most Western societies are now multiracial communities, the political implications of the debate are substantial, and it is hard to consider human populations with the same cold, objective eye as populations of newts or carabic beetles. If we try to do so, human ethnic groups fit rather well into the classical definition of *subspecies* or *geographical races*. As we have said above, population trees closely follow classically identified ethnic groups (Figs. 19.2 and 19.4), which supports the view that human ethnic groups can be considered as geographically differentiated populations. Phenotypic differentiation among them, particularly skin color, is probably adaptive to environmental factors, although some authors [20] postulate that they are mainly the result of sexual selection. Anybody having experienced long-term stays in tropical or equatorial areas, which I have done, can testify that people of African origin are better adapted to such climates. French Guiana, where I have spent 1 year, has not been called a "white people's tomb" for nothing. Indian populations of the Bolivian Altiplano are very well adapted to altitude (the Altiplano is 4000 m high). Studies at the French-Bolivian institute of altitude biology (http://saludpublica.bvsp.org.bo/ibba/), where I have spent 5 years, have shown that the original ethnic group of the Altiplano, the Aymara Indians, are the best adapted. They have a powerful respiratory capacity and make red blood cells very easily (Fig. 19.6).

The Quechuas, who are more recent immigrants (they are the descendants of the Incas and came in the Altiplano area at the 15th century) are less well adapted. Bolivians of direct Spanish ancestry (or other European ancestry) are also poorly adapted. Certain individuals suffer from some pathologies (polyglobulia) even if they were born in La Paz, and have to live in lowland areas. Crossbred people are half-way between Indians and Caucasians for altitude adaptation. It is remarkable that the Aymara became so well adapted in only a few thousand years. This illustrates the very strong phenotypic adaptability of our species to environmental parameters. It is logical to predict that this property involves genetic susceptibility to infectious diseases as well (see below).

Concerning the biological validity of ethnic groups, it can be noted that in the recent special issue of *Nature Genetics* published on this topic [22, 25, 33, 41], the opinions of the different contributors are quite disparate. This shows that there is no consensus among specialists on this

(a)

(b)

(c)

Fig. 19.6. Aymaras (a) and Quechua (b) Indian people in Bolivia. The Aymara woman and her child show typical "Altiplano faces" with red cheeks, indicating a physiological polyglobulia. This is an adaptation to the low oxygen rate that exists at 4000 m. "Altiplano cheeks" are not a specificity of Aymara Indians: European children get them, too (see (c) the author's daughter, second author of Chapter 40). However, native Altiplano people make red blood cells much more easily than imported populations do (a), (b): credit IRD. See color plates.

point. To a large extent, it is a war on words and a battle of definitions. It can be noted that a phylogenetic definition of subspecies or geographical race is not logical for any species, since by definition subspecies do not constitute distinct clades since they are still capable of interbreeding. It is therefore not unexpected that different human ethnic groups are not fully corroborated by strict phylogenetic criteria [25]. Today the validity of the subspecies/geographical race is debated by many, not only for our own species, but also whatever the species considered. This is really a matter of appreciation. Any reasonably trained naturalist knows that subspecies do exist. They result from recurrent, obvious observations and delight collectors of carabic or cetonic beetles.

This debate on the validity of the subspecies concept is a never-ending story (as is the current debate on the species concept; see chapter 20) and could fuel an entire treatise. For the purposes of this book, the important point to be explored is the medical relevance of population/ethnic diversity in the specific case of transmissible diseases.

Fig. 19.7. *Glossina palpalis* (tsetse fly), the major vector of HAT in Western Africa (credit IRD)

19.5.2 Population and Ethnic Diversity with Regard to Transmission and Severity of Infectious Diseases

The selective pressure caused by infectious diseases shows considerable differences among geographical locations, ecological areas, climatic zones, and continents. Pathogens are not equally distributed, and, for example, malaria exerts its selective pressure mainly in tropical areas and almost totally spares northern countries. As noted in Section 19.1, this should be even more closely verified in the case of vector-borne diseases, as geography and climate strongly drive vector distribution as well, and in most incidences there is a high vectorial specificity to given pathogens. For example, human African trypanosomiasis (HAT; formerly called sleeping sickness) is strictly transmitted by tsetse flies. These Diptera of the genus *Glossina* (Fig. 19.7) are found only in subtropical Africa. The selective pressure of HAT is therefore limited to this part of the world. Some authors have noted that the northern limit of tsetse fly distribution follows the southern limit of the penetration of Islam in Africa. It looks as though Arab conquerors had been stopped by HAT.

Different human populations are therefore expected to exhibit different patterns of susceptibility/resistance to different infectious diseases. The data presented below support this view.

It is a classical notion that native Amerindian populations were decimated by the pathogens brought by Europeans. Tuberculosis is a classical example [32]. The differences in AIDS epidemiology between Africa, on the one hand, and North America and Western Europe, on the other hand, have obvious environmental explanations. However, it is now clear that genetic factors also have an impact [14,34], which makes Africans more susceptible to AIDS than Europeans. This is due to allelic frequency differences for a deletion in the CCR-5 chemokine receptor gene. This deletion, which has a protective role, is much more frequent in Caucasians than in Africans. Another viral disease, hepatitis C, also has a pattern of unequal

(a)

(b)

Fig. 19.8. The Fulani ethnic group (a), which exhibits a high level of genetic resistance to malaria compared to neighboring ethnic groups such as the Mossi (b)

distribution of genetic susceptibility among human populations [38]. A very classical example of differential ethnic impact of infectious diseases is the case of malaria in Africans and African Americans [3,26,27]. Balanced polymorphism, due to the selective pressure of malaria, has been fairly well demonstrated for hemoglobin S (sickle-cell anemia). The homozygous genotype *S/S* is 100% lethal in Africa, whereas the heterozygous individuals (*S/A*) have a selective advantage compared with children (*A/A*) who do not have the mutant allele genotype. Heterozygous individuals (*S/A*) are resistant to *Plasmodium falciparum*, the agent of the most malignant form of malaria. This persistence of a deleterious gene by selective pressure of a disease is a case of balanced polymorphism. Sickle-cell anemia balanced polymorphism suggests a high mortality rate from malaria in Africa.

Although their genetic relationships with malaria are less clear, it has been proposed [27] that the high frequency of other pathological traits in African-Americans and Africans are due to a selective advantage they confer in malaria-endemic areas. This would be the case for high blood pressure and iron overload with cirrhosis. It is worth noting that although African-Americans have undergone great genetic admixture from Caucasian populations, they still exhibit very specific genetic polymorphisms linked to malaria selective pressure. Statistical genetic differences between African-Americans and Caucasian Americans are also reflected in their HLA polymorphism (see Section 19.6 below), which is considered to be caused by the selective pressure of infectious diseases. It is probable that major transmissible diseases other than malaria have played and still play a major selective role in Sub-Saharan Africa. HAT and schistosomiasis are possible examples. Today, the HIV epidemic most probably has a drastic selective impact on African populations.

The few examples cited above support the view that ethnic diversity is a parameter to be taken into account in the study of human genetic diversity and susceptibility to infectious diseases. Differences are not limited to the broad groups previously referred to as *grand'races* [42] (Caucasians, Africans, Asians) and seem to act also, if not more, at a fine level. This is conceivable because different selective pressures caused by pathogens and vectors may act at very subtle ecogeographical levels as well. An illustration is the differential susceptibility to malaria among West African ethnic groups. The Fulani ethnic group (Fig. 19.8) exhibits a high level of resistance to malaria compared to other groups, although all live in the same area [28].

Differences between ethnic groups, even at a subtle level, are important to take into account for surveillance and control of infectious diseases and for easier identification of susceptible individuals. However, one has to keep in mind that in the same population there can be considerable individual differences in genetic susceptibility to infectious diseases [1]. Both population and individual levels must therefore be considered in attempts to control infectious diseases.

19.6 OUR GENETIC INHERITANCE HAS BEEN SCULPTED BY INFECTIOUS DISEASES

Haldane [19] was the first to propose that infectious diseases have been the most drastic selective pressure acting on the evolution of the human species over the last 5000 years. In fact, it is probable that the selective impact of infectious diseases is much more ancient. However, epidemics spread more easily in large human populations grouped in big cities than they do in the scattered small populations before the agriculture era. Selective pressure has therefore probably been stronger since the Neolithic era.

A solid indication of the impact of infectious diseases on human evolution is the molecular evolution pattern of the HLA system. Some HLA human alleles have a very ancient coalescence time, previous to the phylogenetic divergence among Catarrhinians (Old World monkeys), and are genetically more closely related to some *Macacus* alleles than to other human HLA alleles. The explanation hypothesized [6] is balanced selection by infectious agents, mainly malaria. Logically, HLA polymorphism is very different between different human populations, for example, between African-Americans and Americans of European ancestry, as the close ancestors of the first group have undergone the selective pressure of malaria. This fact has practical medical consequences, as HLA typing in African-Americans lacked precision before the advent of sequence typing and led to a higher rate of organ transplant failures in this group.

19.7 MAJOR INTERNATIONAL PROGRAMS THAT WILL BOOST OUR UNDERSTANDING OF HUMAN GENETIC DIVERSITY

19.7.1 The Human Genome Project (HGP): Something Like Landing on the Moon

The HGP can be considered as the equivalent of the Apollo program to conquer the Moon for molecular biology. When the required technology (automatic gene sequencing) became available, two rival programs, one that was monitored by a private company (Celera Genomics; http://www.celera.com/) and one that depended on an international consortium of public institutes coordinated by the National Institutes of Health in Bethesda, MD (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml), entered a race that lasted several years and published the entire sequence of the human genome almost simultaneously, although Celera Genomics had a small advance [12]. The results were somewhat surprising, as our species, which is considered (probably with great anthropocentrism) as the most sophisticated product of evolution, has only about 25,000 genes. This is a hard lesson for speculative scientists, who have professed for decades, without the slightest evidence, the dogma of a minimum number of 100,000 genes for humans.

The HGP has provided an invaluable source of information and has already led to the discovery of many genes of interest that are involved in pathological processes [17]. However, it is probable that knowing the whole sequence of the genome will not lead automatically to a thorough understanding of how genes design phenotypes (see Section 19.1). Inferring the contrary is possibly akin to thinking that knowledge of the alphabet makes it possible to thoroughly appreciate the genius of Shakespeare. Another limitation of the HGP is that it gives a dim idea of the whole genetic diversity of our species, as only very few individuals, all of Caucasian origin, were used as donors. This is why the two programs mentioned below were initiated.

19.7.2 A Welcome Enterprise: The Human Genome Diversity Project (HGDP)

After the start of the HGP, it appeared that with only one individual for each part of the genome to be sequenced, one would miss an important parameter: the genetic polymorphism of our species. This was all the more worrisome that the individuals selected for the HGP were all Caucasians. Today, European populations exhibit a strong genetic homogeneity, and much of the overall genetic variability of our species is found in African populations [36]. It was soon proposed [9] that a complementary program to the HGP should be designed, with a survey of a representative sample of human diversity through the analysis of diversified gene sequences. The project, baptized the HGDP focused on those human populations that had remained genetically isolated for a very long time ("native people"), as they are considered to convey more precise information than urban populations, which have been subject to much admixture. The project generated great opposition from many organizations representing indigenous peoples, mainly the World Council of Indigenous Peoples (WCIP) [24]. Although the concern of these organizations about "genetic colonialism" is quite understandable, from a scientific and medical point of view, having a clear idea of human gene sequence polymorphism remains highly desirable. Meanwhile other valuable initiatives have been launched. An African-American Diversity Project has been designed [23]. The specific HLA polymorphism seen in this group is one of its particular interests (see above). The European Union has launched a diversity program in Europe through a network of laboratories stretching from Barcelona to Budapest [24]. Lastly, a Chinese HGDP has already yielded valuable results [10]. Fortunately, the HGDP has now resettled on happier grounds [8]. A total of 1064 lymphoblastoid cell lines have been collected at the Jean Dausset Foundation in Paris, corresponding to 51 populations from all continents. These have already been genotyped with 404 microsatellite markers distributed in the whole genome. However, from the medical point of view, the information garnered will be drastically limited by the fact that data on the samples concern only the gender, population, and geographical origin of the subjects. For ethical reasons, no information will be issued on possible pathological traits. It will therefore be impossible to establish any links between given pathologies (including infectious diseases) and genetic polymorphisms. Limiting information derived from the data from the samples is ethically estimable. However, it is highly desirable to complete the HGDP with more specialized programs, including thorough collections of relevant clinical and biomedical information. This will be beneficial not only for medical purposes but also for our basic understanding of human evolution, as a dominant characteristic is its long-term coevolution with pathogens.

19.7.3 The HapMap Project: Could It Miss Its Target?

HapMap stands for International Human Haplotype Map Project. This huge program is handled by an international consortium [21] whose goal is to determine the genotypes of 10^6 or more sequence variants, their frequencies, and the degree of association between them in four different populations: Africans (only one ethnic group, the Yoruba from Nigeria), Han Chinese, Japanese, and Caucasian Americans. This sample, although it is better than the sample of the HGP (which includes only a few Caucasian individuals) is highly biased. Han Chinese and Japanese are very closely related and therefore should not be separately sampled. The diversity of Africans is considerable and will not be correctly represented by only one ethnic group. Lastly, many ethnic groups are lacking, including Amerindians and Pacific Islanders. The sample of the HGDP is much better (see above). This imperfect sample will have limited drawbacks when looking for those diseases whose genetic background follows the so-called common disease/common variant (CDCV) hypothesis, in which the genetic factors involved are evenly distributed among populations. If the opposite hypothesis prevails (the multiple rare variant [MRV] hypothesis), the HapMap project will lack resolution [33]. Unfortunately, available data do suggest that genetic traits related to susceptibility to infectious diseases are population linked and therefore follow the MRV pattern. It can therefore be feared that the HapMap project will be of limited help in this specific biomedical field of research.

19.8 CONCLUSION

Our species has limited genetic variability because we are a "young" species: our common mothers and fathers lived only 100,000 years ago [36]. At the same time, we have a notable genetically driven phenotypic polymorphism resulting from strong selective pressures acting differently on separate geographical populations [40]. Although direct evidence is still limited, strong circumstantial evidence clearly shows that susceptibility to infectious diseases has a genetic component acting both on individual and population/ethnic levels. This makes *pharmacogenetics* [13] a field that has a promising future: the administration of a given drug should be adapted to the genetic make-up of individuals and populations. When the

population level is considered, it is more informative to address a fine level (e.g., Fulani vs. Mossi; see Fig. 19.8) rather than the major subdivisions classically distinguished by physical anthropology [42]. However, when analyzing multiethnic populations from a medical point of view, it would be misleading to neglect the ethnic component, at least for research.

The indisputable impact of this genetic component of human susceptibility to infectious diseases should not lead us to neglect two other major parameters acting on the transmission and severity of these human scourges: (i) the role played by the pathogen's genetic diversity (the theme of Chapter 20) and of the vector's polymorphism when vector-borne diseases are concerned (see Chapter 25); (ii) the crucial role of environmental parameters, including socioeconomic features (see Introductory chapter).

ABBREVIATIONS

AIM:	Ancestry informative marker
HapMap:	International Human Haplotype Map (project)
HAT:	Human African trypanosomiasis (formerly sleeping sickness)
HGDP:	Human Genome Diversity Project
HGP:	Human Genome Project
HLA:	Human leukocyte antigens
MLEE:	Multilocus enzyme electrophoresis
SNP:	Single-nucleotide polymorphism

GLOSSARY

Allele: Different molecular forms of the same gene.

Allelic frequency: The ratio of the number of a given allele to the total number of alleles in the population under survey.

Alu insertion sequence: Dispersed repeated DNA sequences in the ht human genome, consisting of about 300 bp in approximately 3×10^5 to 5×10^5 copies, corresponding to about 5% of the human genome. Each segment is about 300 bp long. These sequences are easily transposable. They are specifically cleaved by the restriction enzyme Alu I.

Ancestry informative marker (*AIM*) [31]: AIMs are the subset of genetic markers that are different in allele frequencies across the populations of the world. Most polymorphisms are shared among all populations, and for most loci the most common allele is the same in each population. An ancestry informative marker is a unique set of genetic markers that occurs mostly in particular founder population sets but may also be found in varying levels across all or some of the populations found in different parts of the world (definition communicated by DNAprintgenomics from the website of DNAprintgenomics, with the kind authorization of the company).

Balanced polymorphism/balanced selection: Genetic polymorphism that persists in a population because the heterozygotes for the alleles concerned have a higher fitness than either homozygote.

Bootstrap analysis: In phylogenetic analysis, generation of pseudoreplicate data sets by randomly sampling the original character matrix to create new matrices that are of the same size as the original. The frequency with which a given branch is reproduced by this randomization procedure is recorded as the bootstrap proportion. These proportions can be used as a measure of the robustness of individual branches in the tree. A bootstrap value of 95 for a given branch means that this branch has been found 95 times out of 100 by the procedure.

Clade: Evolutionary lineage defined by cladistic analysis. A clade is monophyletic (it has only one ancestor) and is genetically isolated (which means that it evolves independently) from other clades.

Cladistic analysis: A specific method of phylogenetic analysis based on the polarization of characters that are separated into ancestral (plesiomorphic) and derived (apomorphic) characters. Only those apomorphic characters common to all members of a given clade (synapomorphic character) are considered to have a phylogenetic value. For example, feathers are specific of the clade "birds" and are featured by all birds. They are a synapomorphic characters of that clade.

Coalescence time: Time elapsed between the common ancestral copy (one gene in one individual) and two or more copies of a given gene at the present time.

Ethnic group: A group whose common identity is based on racial/and or cultural association.

F_{is}: See *F*-statistics (Box 1).

Founder effect: When a small subsample of a larger population settles as a distinct population, its genetic diversity might be only a small part of that of the original population. The French Canadians were founded by only a few thousand French, most originating from a few French regions. Their original diversity (and the diversity of their last names) was therefore lower than that of the entire French population. This explains why some pathological traits (amyotrophic lateral sclerosis) are more frequent in Quebecois than in the present French population.

F_{st}: See *F*-statistics (Box 1).

Gene: A DNA sequence coding for a given polypeptide. More broadly speaking, any given DNA sequence.

Gene sequence (*or genetic sequence or DNA sequence*): Can be compared to a series of letters representing the primary structure of a real or hypothetical DNA molecule or strand. The possible letters are A, C, G, and T, which correspond to the four nucleotide subunits of a DNA strand (adenine, cytosine, guanine, and thymine). This coded sequence represents basic genetic information. A DNA sequence may code for

proteins. In this case, it directly monitors the succession of amino acids that constitute the primary structure of the protein. Some other sequences have no known coding function.

Genetic distance: Various statistical measures inferred from genetic data, estimating the genetic dissimilarities among individuals or populations. Genetic distances can be based on percentage of band mismatches on gels (the cases for markers such as MLEE or RAPD) or allelic frequency differences or percentage of sequence divergence.

Genomics: Molecular analysis of the whole genome of species.

Genotype: Genetic constitution of a given organism; *cf.* phenotype.

Geographical race: *cf.* subspecies.

Heritability: Proportion of variation in a trait among individuals in a population that can be attributed to genetic effects.

Human leukocyte antigens (*HLA*): Cell surface proteins detected by blood testing that exhibit considerable diversity among individuals. Also called histocompatibility antigens or tissue antigens because organ recipients and donors should have compatible HLA genotypes; otherwise, the transplanted organ is recognized as nonself (foreign) and is rejected. This set of genes is involved in the presentation of antigenic peptides to the immune system and plays a major role not only in tissue compatibility but also in infectious processes.

Insertion sequence (*IS*): A small bacterial transposable element, approximately 1000 bases long, with short inverted repeated sequences at its ends.

Intron: A gene region that is not translated into a protein sequence. In coding genes, introns are interspersed with coding regions called exons.

Isoenzymes: Protein extracts of given biological samples are separated by electrophoresis. The gel is then processed with a histochemical reaction involving the specific substrate of a given enzyme. This enzyme's zone of activity is then specifically stained on the gel. From one sample to another, migration differences can appear for this same enzyme (see Figs. 19.1 and 19.3). These different electrophoretic forms of a single enzyme are referred to as isoenzymes or isozymes. When given isoenzymes are driven by different alleles of a single gene, they are more specifically referred to as alloenzymes or allozymes. Differences in migration result from different overall electrical charges between isoenzymes. Overall electric charges are a resultant of the individual electric charges of each amino acid (AA) of a given enzyme. The AA sequence is the direct result of the DNA sequence of the gene that codes for this enzyme. It is therefore considered (and verified) that isoenzyme polymorphism is a faithful reflection of the genetic polymorphism of the organism under study.

Linkage disequilibrium: Nonrandom association of genotypes occurring at different loci.

Microsatellite A short DNA sequence of DNA, usually 1–4-bp long, that is repeated together in a row along the DNA molecule. In humans (Chapter 19), there is great variation from person to person (forensic use of individual identification) and among different populations in the number of repeats. Numbers of repeats for a given locus define microsatellite alleles. There are hundreds of places in human DNA and in most other species that contain microsatellites. Microsatellites are fast-evolving markers, with a high resolution level and are found in many other organisms, including pathogens (widely used in molecular epidemiology; see Chapter 20).

Multilocus enzyme electrophoresis: Isoenzyme analysis based on the analysis of a broad range of enzyme systems. Each enzyme system corresponds to one or several genetic loci. Strains that share the same MLEE profile are referred to as zymodemes (parasitology) or electrophoretic types (bacteriology).

Natural selection: Process first described by Charles Darwin that favors certain genotypes to the detriment of others over generations. It is entirely driven by the interaction of an organism with the environment.

Neutral gene neutral polymorphism: A gene/genetic polymorphism that does not undergo natural selection.

Phenotype: All observable characteristics of a given individual or a given population apart from the genome. The phenotype is not limited to morphological characteristics and includes, for example, physiological parameters (blood pressure, etc.) or biochemical ones (level of cholesterol, etc.). The phenotype is produced by the interaction between genotype and the environment.

Phylogenetics: A branch of genetics that aims at reconstructing the evolutionary past and relationships of taxa or of separate evolutionary lines.

Pulse field gel electrophoresis (PFGE): Separation of large DNA fragments by a particular electrophoresis technique using alternately pulsed, perpendicularly oriented electrical fields. Strains that share the same PFGE are referred to as pulse types. In the case of bacteria, the large DNA fragments result from the action of a low-frequency cutter (a bacterial endonuclease whose restriction action has a low frequency) on the bacterial chromosome. It is therefore a specific case of RFLP. With parasitic protozoa (*Trypanosoma* and *Leishmania*) and yeasts, the large DNA fragments correspond to entire chromosomes (molecular karyotype).

Race: Population characterized by a set of common genetical features [18]; *cf.* ethnic group, subspecies, geographical race.

Recombination: Reassortment of genotypes occurring at different loci.

Sexual selection: Selection that promotes traits (e.g., the peacock's tail) that increase success in mating.

Sickle-cell anemia: A generally lethal form of hemolytic anemia observed in individuals who are homozygous for the autosomal, codominant gene H^s. The red cells of these individuals

contain an abnormal hemoglobin, Hb^s. These red cells exhibit a reversible shape alteration when the oxygen concentration in the plasma falls slightly and develop a sickle-like form. These pathological red cells have a shortened lifetime. Approximately 0.2% of African-American babies suffer from sickle-cell anemia.

Single-nucleotide polymorphism (SNP): Polymorphisms or one-letter variations in the DNA sequence. SNPs contribute to differences among individuals and populations. Most of them have no effect, others cause subtle differences in countless features, such as appearance, whereas some affect the risk for certain diseases. Widely used as high-resolution population markers.

Spacer DNA: In eukaryotic and some viral genomes, untranscribed DNA segments that flank functional genetic regions or cistrons.

Subspecies (*also called geographical race*): Geographical populations of the same species that exhibit distinct genetically based phenotypic traits making it possible to distinguish most individuals of one population from most individuals of another population.

Synonymous mutation: Mutation that gives change in DNA but no change in protein due to redundancy of the genetic code.

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CHAPTER 20

Molecular Epidemiology and Evolutionary Genetics of Pathogens

M. Tibayrenc

20.1 INTRODUCTION: MOLECULAR EPIDEMIOLOGY (ME) AND EVOLUTIONARY GENETICS ARE INSEPARABLE

This chapter is willingly medically oriented. It aims at illustrating the practical use of modern molecular tools for biomedical research and applications dealing with pathogen identification. However, the reader should be aware that the boundary between applied and fundamental research is sometimes artificial. In the present chapter, I will show that interpreting ME data (applied research) without the help of evolutionary genetics concepts (basic research) is (i) boring and (ii) often grossly misleading. As an example, if I take some DNA from all the authors of this book (there are many of them), and I perform any typing method on this sample (for example, RAPD), I can have this set of molecular data computer analyzed. The computer will always manage to issue a tree of this data set, or more exactly, a dendrogram. However, this tree is by no means a phylogenetic tree, as the authors of this book are not different taxa or evolutionary lineages. They are only a sample of the individual genetic variability of our species. An intuitive, visual reading of such a dendrogram could lead to assimilating its branchings to clades, which would be quite misleading.

The molecular polymorphism of pathogens has been shaped by evolution. It is therefore logical to interpret this in terms of evolutionary genetics, as this discipline has been explicitly designed for this goal, thereby making a clear-cut distinction between applied and basic research artificial. Still, the fact remains that biomedical research has specific goals and requires precise, operational answers. Medical doctors, veterinarians, health professionals, and decision makers need to know, or need to know that we do not know. For example, in the context of avian flu, it is crucial to know whether a given flu virus is H5N1. This need for black and white answers is not obligatorily the case for evolutionary research, which can and should handle a strong dose of fruitful, speculative exploration.

Based on the approach long supported by our group [76,77,86], I propose here a number of common approaches valid for all kinds of pathogens, including pluricellular (worms) and unicellular (eukaryotic: parasitic protozoa and fungi; prokaryotic: bacteria and viruses) pathogens. Of course, each of these groups raises specific problems. Parasitic protozoa, microfungi, and bacteria have much in common. This is less the case when worms and viruses are considered. However, many things remain common among all categories of pathogens (the problems for defining species and strains, for example) and many molecular tools can be used for all of these pathogens (e.g., gene sequencing). Moreover, the goals and mission of ME are similar whatever the pathogen considered (see below).

A comparative approach considering all categories of pathogens makes it possible (i) to draw up the general laws that govern pathogen molecular evolution and the common rules of ME and (ii) at the same time, to underscore the specificities of each case.

It should also be emphasized that the concepts and methods exposed here are valid for pathogens of *medical*, *veterinary*, and *agronomical relevance*. In addition, the distinction between pathogens of medical or veterinarian relevance is sometimes arbitrary, as many species such as tuberculosis and Chagas disease infect both humans and animals. Such cases increase the relevance of ME, which is the choice method for identifying the possible animal reservoir of a human disease. The basic approach here is to evidence that the putative reservoir harbors the same pathogen species and the same strains that infect humans.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

20.1.1 ME Mission Statement

ME refers to "the various biochemical and molecular techniques used to type and subtype pathogens" [13]. ME's mission statement thus defined is quite clear: it is to *identify* pathogens at the relevant levels: species, subspecies, strains, clones, and genes of interest [83].

Considering the need to rely on the concepts of evolutionary genetics to reach this goal, a more detailed definition of ME has recently been proposed [83].

1. Definition, identification, and tracking of pathogen species, subspecies, strains, clones, and genes of interest by means of molecular technology and evolutionary biology.
2. Evaluation of the impact of a pathogen's genetic diversity on its relevant medical properties such as pathogenicity or drug resistance (downstream studies).

This definition implies several important points: (i) before identifying species, subspecies, strains, and clones, it is indispensable to clarify these concepts, never a simple task, especially in the case of pathogens (see Section 20.4). Evolutionary biology is an indispensable tool for that. (ii) Limiting oneself to identification leads to a rather narrow definition of ME. Exploring the extent to which the genetic diversity of pathogens influences the properties of interest to medical personnel ("downstream studies") demonstrates the wealth of ME. The minimal way to take this into account is as follows. Let us say (dream) that somebody discovers an efficient vaccine against malaria. It would be indispensable to verify that this vaccine works against a set of strains that is representative of the whole genetic variability of the parasitic species involved. Similarly, if somebody found a more efficient and less-toxic drug against Chagas disease than Rochagan®, it would be crucial to test it against various strains of the parasite. In the case of Chagas disease, this would be all the more desirable given the impressively high genetic diversity of this parasite (see below). This point is neglected by most applied studies dealing with pathogens.

Lastly, a strong commitment of ME is to design technical tools that are relatively easy to use and reasonably inexpensive, mandatory for these tools to be used routinely. The cost dimension is especially crucial when health care professionals from developing countries are concerned (see Chapter 38).

20.3 THE MODERN HYPERMARKET OF MOLECULAR TECHNOLOGIES

More than the golden age of genetics [79], our time is *the golden age of genetic technologies* [80]. This is greatly boosted by the private medical industry, as infectious diseases have become a major commercial market today (see Chapter 38).

It is not the goal of this chapter to establish a catalog of the many technologies available, even less so because every year sees the birth of several new ones. I will instead try and outline a few guidelines for selecting appropriate techniques to be used in specific cases.

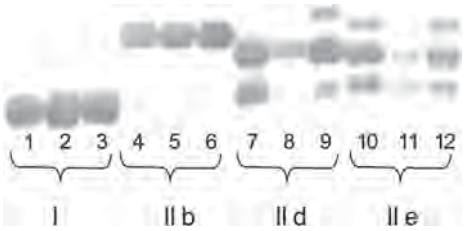

Fig. 20.1. An isoenzyme gel for the genetic locus Glucose phosphate isomerase (Gpi) showing the genetic polymorphism of different genetic subdivisions (discrete typing units; see Box 20.3) of *T. cruzi*, the protozoan parasite responsible for Chagas disease in Latin America. [69]. Although isoenzymes are an old-fashioned marker, they are able to sharply delimitate *T. cruzi* DTUs with highly recognizable patterns.

20.3.1 Four Key Starting Points

(i) *There are no good and bad markers.* Modern technology has flooded us with a wealth of sophisticated markers. However, it would be a mistake to think that the best ones are the latest ones to come out on the market. Some cherished old tools such as isoenzymes (see Chapter 19), widely used since the 1960s, still convey irreplaceable information in routine identification and remain a type of gold standard for ME and population genetics (Figs. 19.1 and 19.3).

What should be made crystal clear is that *there are not good and bad markers*; rather there are only markers that are best suited to answering a given question. Each marker has its labor and monetary costs, specific properties, level of resolution, and drawbacks, all of which must be weighed before a given marker is selected for a ME study. Isoenzymes are adequate to identify the species *Trypanosoma cruzi* and its main genetic subdivision, different Leishmania species, and in general to evidence the genetic variability of most species of pathogens and other organisms (generalist marker, see below) with a fine level of resolution (although not as fine as other markers such as microsatellites). However, they are of little use in the case of *Mycobacterium tuberculosis*, the agent of tuberculosis (see Chapters 1 and 5), for this bacterium exhibits very limited variability for housekeeping genes and hence for those genes that govern isoenzyme variability. Moreover, isoenzymes require that the pathogen be cultured (except in the case of big worms). Lastly, their portability is limited. Results may not be easy to exchange between different laboratories.

(ii) *ME, according to its fundamental goals, considers mostly population levels* and hence requires *extensive samples* that are representative of the entire ecogeographical range of the species under study. It is therefore indispensable to keep a balance between the development of expensive, sophisticated technologies and the need for informative samplings. This is especially true with the huge international

programs sequencing entire genomes. Following the launch of the Human Genome Project (see Chapter 20), many projects were initiated, aiming at sequencing the whole genome of many major pathogens (including, *M. tuberculosis*, *Plasmodium falciparum*, the agent of the most malignant form of malaria, *Trypanosoma brucei*, the agent of human African trypanosomiasis, and many others). This genomic approach is now indisputably necessary, even if less heavy technologies (MLEE, RAPD, RFLP, PCR, etc.) remain extremely useful.

Sequencing a single strain of a given species gives a strongly biased, mutilated picture of the total genomic make-up of that species in its entire ecogeographical range. In its very essence, whole sequencing is a *typologist* approach, that is, the approach that considers that each species is well represented by an ideal type – the specimen used for the first description – and that all variability around this type can be ignored. Traditional taxonomy is definitely typologist. When a new species of beetle is to be described, the entomologist who discovered it deposits it in an certified museum (in France the Natural History Museum in Paris) a "type," which will be the reference for all entomologists worldwide to see what this new species looks like. In pathogen sequencing programs, there is a risk that specialists neglect the genetic variability of the species that have been sequenced.

The challenge here is to build a hybrid discipline, a combination of population genetics and genomics, thus favoring the birth of *population genomics*.

(iii) *Computing these data is a major challenge*. A consequence of the need for both genomic and population approaches is the computational burden associated with it. The analytical methods and availability of software do not sufficiently allow for thorough analyses of these floods of data. This is a major challenge for the emerging field of bioinformatics.

(iv) *Preference for easy-to-use tools*. As already stated, ME's mission statement is to address practical goals: technologies that are expensive, time consuming, and usable solely by sophisticated laboratories should not be the only ones on the market. This demand is especially true in developing countries. Molecular technologies that are problem solving as well as affordable must be developed. One must remember that the scourge of infectious diseases strikes southern countries first (see Chapters 35, 37, 38, and 40).

20.3.2 Classification of Markers

Markers can be classified according to various criteria. These are mentioned below.

20.3.2.1 Routine ME or advanced research? There is actually a continuum between the fields of ME and advanced research. ME in its present state of development is never as routine as the currently used serological tests such as ELISA or indirect immunofluorescence. On the other hand some sophisticated techniques, even if they are reserved for advanced teams, may convey a great deal of epidemiological information. The fact remains that a distinction at this level can be very clearly made. The following techniques are more widely used in routine identification and epidemiological tracking, with a limited technological added value: AFLP, ERIC, MLEE, PFGE, RAPD, RFLP, and ribotyping. Conversely, other markers remain more in the domain of advanced research limited to sophisticated environments: gene sequencing, MLST (Box 20.1), and microarrays (Box 20.2). It should be noted that PFGE is a routine tool in bacteriology and more a research technology used to investigate parasites and yeasts (see Glossary).

20.3.2.2 Level of resolution required: The Notion of Molecular Clock Let us consider a phylogeneticist trying to establish the overall molecular evolution of the entire genus *Trypanosoma*, those parasites that transmit Chagas disease and human African trypanosomiasis, among others. This kind of study is thoroughly discussed in Chapter 17. Consider an epidemiologist who wants to identify the culprit clone of *Staphylococcus aureus* that has contaminated a whole intensive care unit at the hospital. Chapter 1 exposes similar problems with *M. tuberculosis*. Obviously, the two problems address different evolutionary scales, and hence require molecular tools that have a different magnifying power. This power of resolution is conditioned by the molecular clock of the genomic region that governs the variability of the marker. For a given set of stocks, a marker with a fast molecular clock reveals more different MLGs than a marker with a slow molecular clock. A fast marker is required for detailed ME studies on limited time scales and space, such as the study on the *S. aureus* clone described above. On the other hand, it will not be adequate in the case of a bird's-eye-view phylogenetic study on higher levels of evolutionary divergence, such as the above-mentioned *Trypanosoma* phylogenetic study. The trees will hide the forest, so to speak, and all strains will appear as equally distant from each other, with the maximum value of genetic distance (Fig. 20.2).

20.3.2.3 The PCR revolution: culture-free versus non-culture-free procedures The PCR procedure has revolutionized ME, as it can skip the culture step, which introduces a culture bias [78]. In the frequent case of the occurrence of different multilocus genotypes (MLGs) in a given isolate, it is possible that the culture medium selects some MLGs to the detriment of others. It is therefore relevant to distinguish between markers that require a culturing step (multilocus enzyme electrophoresis [MLEE], random primed amplified polymorphic DNA [RAPD], microarrays, etc.) and markers relying on the PCR technology, which are not biased in this way. A related advantage of PCR is its sensitivity, as it is virtually able to amplify the DNA of a single cell. A last advantage is its specificity, provided that primer designing is done carefully. All these advantages concern only those PCR protocols that use specific primers, not the other methods based on randomly selected primers (amplified fragment length polymorphism [AFLP], RAPD).

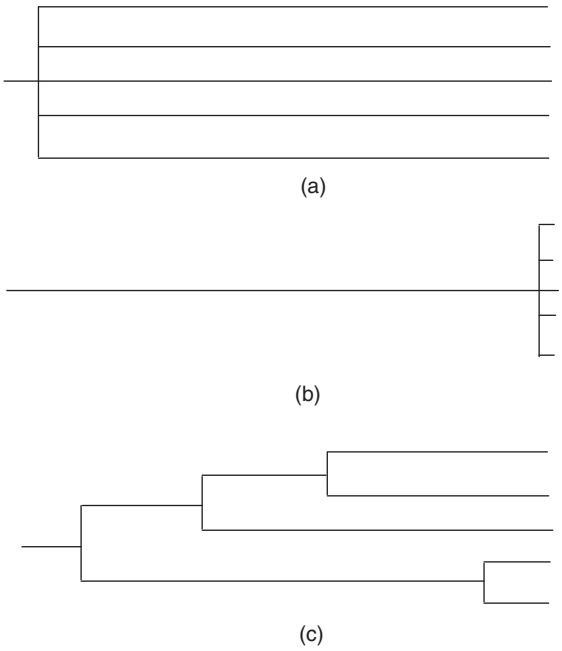

Fig. 20.2. Impact of the molecular clock of the marker used in phylogenetic trees for the same set of strains. (A) Tree derived from a molecular clock that is too fast (rake-like tree). (B) Tree derived from a molecular clock that is too slow (all strains are genetically identical). (C) A convenient molecular clock (the tree shows clear hierarchizations) (after [85]).

20.3.2.4 Generalist and specialist markers Another relevant distinction should be made between specialist and generalist markers [78]. Specialist markers work for only one species or one group of species. For example, RFLP typing by hybridization with the IS6110 insertion sequence [74] can be used only with bacteria of the *M. tuberculosis* complex, as this IS is present only in this group of species. By nature, interspecies comparisons cannot be made with specialist markers. For example, a comparison of the overall genetic diversity of *M. tuberculosis* and *Escherichia coli* is not feasible with IS6110. PCR typing with REP and ERIC repetitive elements [54] do not work for eukaryotic pathogens, as these elements exist in bacteria only. They are less specialized than IS6110 but are not really generalist markers. Generalist markers are usable for any species, which is the case for MLEE (Fig. 20.3) and RAPD.

20.3.2.5 Multilocus markers Another important distinction lies between those markers that allow the identification of distinct genetic loci or genomic regions (MLEE, RAPD, etc.) and those that provide only overall genetic profiles (e.g., genetic black boxes) corresponding to the activity of an unknown number of loci (there could be many) that cannot be distinguished from each other (e.g., RFLP, pulse field gel electrophoresis [PFGE]). This distinction is important, as the analysis of distinct loci is required for some population genetic statistics such as linkage disequilibrium (LD) (see below).

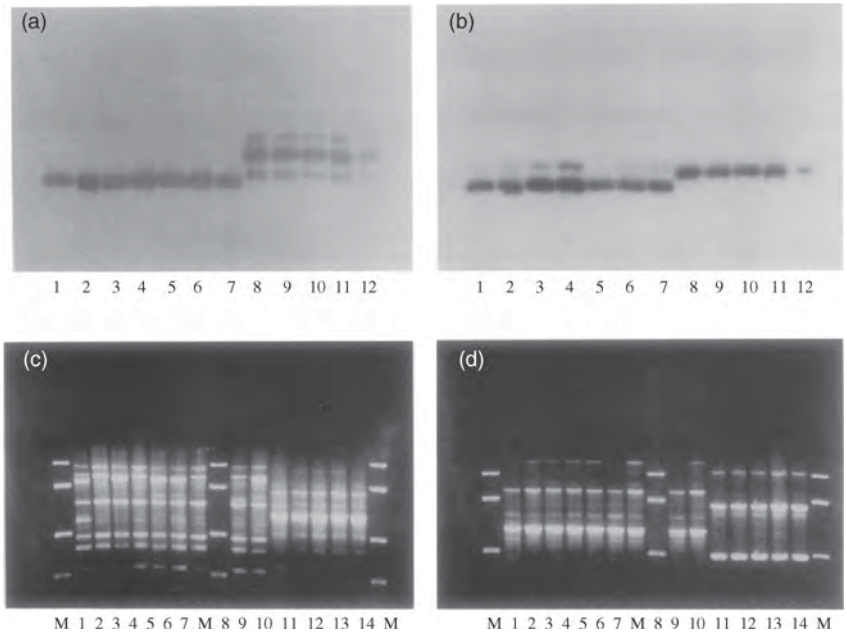

Fig. 20.3. (after [89]) Four electrophoretic experiments, two MLEEs with two different isoenzyme loci (top), two RAPDs with two different primers (bottom) for a set of stocks of *T. cruzi*, the parasite that causes Chagas disease. The same stocks are surveyed on the four gels, but RAPD gels have two additional stocks. On the four gels, only two main genotypes (DTU 1 and DTU 2; see below and Box 20.3) are distinguishable; lines 1–7 and 8–12 for MLEE, lines 1–9 and 10–14 for RAPD. M lines on RAPD gels are molecular weight ladders. Genotypes DTU 1 are systematically associated together, and the same is true for genotypes DTU 2. Crossed genotypes, which would be the result of genetic recombination (e.g., gel A line 1/gel B line 8 + gel C line 1 + gel D line 10), have never been observed on more than 600 *T. cruzi* stocks analyzed by our team until now [8].

TABLE 20.1 Markers Currently Used in Molecular Epidemiology (see Glossary) (After [85])

	Marker							
Properties	MLEE	RAPD	AFLP	RFLP	PFGE	Microsatellites	Microarrays	MLST
Culturing	Yes	Yes	Yes	Yes	Yes	No	Yes	No[a]
Analysis of distinct loci	Yes	Yes	Yes	No	No	Yes	Yes	Yes
Homoplasy	++	++	++	++	++	+	+/−	–
Cost	+/−	+/−	+/−	+/−	+/−	+/−	+++	+++
Labor	+	+	+	+	+++	+	+++	+
Portability	+/−	+/−	+/−	+/−	+/−	+/−	++	+++

[a]If sequences are obtained by PCR.

20.3.2.6 Levels of homoplasy Certain molecular characters that are common to different strains actually originate from other causes than common ancestry. For example, identical isoenzyme bands sometimes correspond to different proteins whose overall electric charge does not differ. It is considered that MLEE reveals about 30% of point mutations [60]. Isoenzyme homoplasy can be compensated by using a sufficient range of loci. Another frequent case of homoplasy is reverse mutations.

Homoplasy mimics genetic recombination. Indeed, if different strains share a given common molecular character, it looks as though one strain had transferred the concerned gene to the other. This also leads to low resolution.

Complete sequences show a minimal level of homoplasy by comparison with other markers; however, the risk still exists, either by reverse mutations or parallel evolution.

Detailed techniques on the various markers available have been described in many review papers (see, e.g. [54,78]). Several of them, which have been cited in this chapter and Chapter 19, are listed in the Glossary. Table 20.1 gives the advantages and drawbacks of some widely used markers.

Multilocus sequence typing (MLST, Chapter 29) and microarrays whose use is presently growing in the field of ME, deserve to be developed in greater detail (see Boxes 20.1 and 20.2).

BOX 20.1: MULTILOCUS SEQUENCE TYPING (SEE CHAPTER 30)

MLST [44] is a highly standardized approach based on the sequencing of 450-bp parts of seven housekeeping genes. It has been widely used for a high number of bacterial species and some yeasts [91]. The main advantage of MLST is it perfect portability, as sequences can be simply emailed between laboratories. Strains that share the same combination of alleles are referred to as *sequence types* (ST). Strains that share seven of seven alleles: *consensus group*; strains that share six of seven alleles: *single-locus variants* (SLV); strains that share five of seven alleles are *double-locus variants* (DLV). SLV and DLV: *clonal complexes*.

Comments:

1. Even if several genes are sequenced, only a limited part of the genome is analyzed.
2. The use of MLST for ME does not fully take advantage of the resolution power of gene sequencing, as the allele, not the point mutation, is taken as the unit of analysis, and levels of phylogenetic relationships are determined according to the ratio of identical alleles. A nonidentical allele is a black box that covers highly different levels of base pair differences (from 1 to the total sequence). The significance of the number of base pair differences is very different in highly recombining species (in which several base pair differences may be due to gene exchange) and predominantly clonal species (in which they reflect evolutionary divergence). This point is not explored by MLST#.
3. Only point mutations are taken into account. Other evolutionary events that may play an important role in pathogen evolution (transpositions, inversions) are not seen, contrary to other typing methods (PFGE and RAPD, for example).
4. MLST considers only one category of genes (housekeeping genes), which may correspond to specific evolutionary patterns and may undergo selective pressure [56]. There is therefore no guarantee that the variability explored is representative of the whole genome. Other typing methods (RFLP, RAPD, and PFGE) do not have this bias.
5. The resolution power of MLST is sometimes lower than more traditional methods such as RFLP. This led the promoters of the method to advise completing the basic MLST approach with the use of a few highly variable genes (antigen genes).
6. Interpretation for diploid, and even more, aneuploid species is difficult, as for a given gene in a given individual, in the case of diploids, there are two copies (alleles) and there is a variable number of copies in the case of aneuploid. Bacterial cloning is therefore mandatory to pick up all copies.

7. Lastly, although automatic sequencing has made important progress, the sequencing of several genes and interpretation of the data still confines MLST to rather sophisticated environments. The design of adequate software (eBURST) [68] will be precious to make this approach a routine one.

BOX 20.2: MICROARRAY (SEE INPRESS [53])

Microarrays are popularized under the names of gene chips or DNA chips. Microarrays are composed of large numbers of molecules (often, but not always DNA) distributed in rows in a tiny space. When DNA molecules are used, they play the role of probes for the DNA of the organism being investigated, to see whether the genes present on the reference sample (the one plotted on the chip) are also present on the sample under study.

Microarrays also allow scientists to study gene expression by showing a snapshot of all the genes that are expressed in a given cell at a particular time (transcriptome).

Their use for ME (pathogen identification and characterization) is growing fast, although it is not a routine diagnostic tool yet [30]. For genotyping, the microarray harbors thousands of short DNA sequences (probes) and a highly purified DNA sample of the strain under study is hybridized with them. Only those genes that are present in the strain will hybridize, and the strength of hybridization depends on the sequence similarity between the reference sample and the sample being studied. Microarrays are powerful for screening complex samples (many genes of a given strain, or complex samples composed of several strains or even several species).

20.4 SURVIVAL KIT FOR EVOLUTIONARY GENETIC INTERPRETATION

It is a challenge to simplify the complex notions of evolutionary biology to make them accessible to nonspecialists without distorting them. However, this enterprise is sorely needed. When I try to sell ME to non-evolutionists (e.g., doctors in a hospital), I always notice that they fall asleep at the very instant I pronounce words such as "synapomorphic character". The modest goal of the present section is to give the nonspecialist a sufficient backdrop against which the main concepts can be understood, thereby making it possible to go beyond empirical descriptions and a simple counting of banding patterns where ME is concerned.

20.4.1 ME's Full Task

The basic assumption of ME is that in natural pathogen populations, there are individualized evolutionary units that are stable and identifiable using adequate molecular tools. The pathogen world is *discontinuous*, *even if its subdivisions are not always clear-cut* [83,84] (see the discussion on the species concepts below). These subdivisions, their discreteness, and their stability are conditioned by evolutionary forces. This explains why evolutionary biology is relevant for clarifying ME data.

Given a particular species whose genetic diversity is not completely known, the following questions have to be answered before ME can be routinely used:

1. What is the theoretical base used for the definition of the *species* under study (see below)? What are its genetic boundaries, if any? Does it correspond to a discrete evolutionary unit that can be identified by specific characteristics?
2. Are there additional discrete subdivisions within the species, themselves characterizable by specific markers?
3. What is the stability over space and time of the MLGs that compose the species?
4. What is the total amount of genetic diversity of the species?
5. What is the entire ecogeographical range and host range of the species?

Once these questions have been answered, one has a convenient framework for species, subspecies and strain typing, the study of relevant genes, and the analysis of the genetic command of the relevant medical properties of the species (pathogenicity, resistance to drugs).

20.4.2 Two Complementary Tools for ME: Population Genetics and Phylogenetic Analysis

The principle of population genetics is to give a snapshot of the population structure of a given species or population at a given time, with specific statistics that measure gene flow and gene evolution in natural populations. The goal of phylogenetic analysis is more ambitious. It is to reconstruct the past of a given species at an evolutionary scale (thousands or millions of years). The separation between the two is sometimes more didactic than real. Phylogenetic methods can be used to evaluate certain population genetic parameters such as the mating system of the species (see below).

20.4.2.1 Population genetics Several parameters influence the population structure of a species: the main being the genetic drift, natural selection, and the reproductive system. When the specific goal of ME is considered (identifying and characterizing the relevant units of analysis in natural populations of pathogens), the last parameter has a predominant and specific role. In the last 25 years, it has led to many discussions that have not been settled (the *clonality/sexuality debate*). Indeed, microbes have strange mating behaviors that are disconcerting for normal sexual beings such as humans.

20.4.2.1.1 Asexology of microbes The main consequence of sexuality is genetic recombination, which shuffles genotypes occurring at different loci. Consequently, *frequent sexual processes render MLGs ephemeral and useless for epidemiological tracking*. In this case, only individual genes are stable entities that can be traced. This is a crucial point in ME. Considering a priori MLGs (strains) as stable entities could be grossly misleading in highly recombining species such as *P. falciparum*, the parasite that causes the most malignant form of malaria, or *Helicobacter pylori*, the bacterium that provokes gastric ulcer. Lack or scarcity of recombination is statistically evaluated by LD, which causes the genotypes at different loci to be transmitted through generations as blocks of genes without reassortment. The notion of LD can be simply explained by the following example. If I know the AB blood group of an individual (e.g., he or she is A, which can correspond to either A/A or A/O genotypes), this gives me no information on what his or her rhesus group is Rh(+) or Rh (–). Indeed, ABO and rhesus are commanded by independent genetic loci that recombine at each generation. They show no LD. If the human species was asexual, ABO and Rh genotypes would be transmitted together as a whole without recombination. This is what happens in *T. cruzi*, the agent of Chagas disease (Fig. 20.3).

LD can be measured by various statistical methods [6,46,88], which all are based on the same principle. They take the null hypothesis that genotypes recombine at random among loci. They evaluate what would be the MLG distribution and frequency under these panmictic expectations. Let us consider the following numerical example, the simplest possible one. Two pathogen strains are surveyed at two genetic loci, A and B. At each locus, two different genotypes, 1 and 2, are observed, and each has an observed frequency of 0.5. The frequencies of the individual genotypes A1, A2, B1, and B2 are hence all 0.5. In this case, the *expected* frequency *under panmictic assumptions* of the composite genotype A1/B1 is $0.5 \times 0.5 = 0.25$. This is the expected probability for two independent events to happen together. Thus, the expected frequencies of the other combinations (A1/B2, A2/B2, and A2/B1) are also 0.25 each. Now if the observed frequency of A1/B1 and A2/B2 is 0.5 each and logically, the observed frequencies of the other possible combinations, A1/B2 and A2/B1 is 0, this is a manifestation of a total LD between A1 and B1 on one hand, A2 and B2 on the other hand. Such departures can be measured by a simple chi-square test if sample sizes are large enough, or with more specialized tests relying mainly on computer randomization procedures (Monte Carlo simulations) [46,88].

If the number of loci surveyed increases, the procedure is still the same. For example, if 10 loci, A–J, are studied, with two genotypes (A1, A2, . . ., J1, J2) of equal frequency (0.5) at each locus, the frequency of any composite genotype will be only $[0.5]^{10}$. This makes this approach extremely powerful: indeed studies involving 15–20 different loci are commonplace, and the expected frequency of each individual MLG becomes very low. The mere repetition of MLG is therefore quite improbable, and becomes in itself a telling indication of departure from panmixia. LD could be explained by the classical cause of spatial proximity of two genetic loci on the same chromosome, inhibiting crossing over between them. However, it is considered that in natural populations, the probability that this parameter can account for a strong LD occurring at many loci is negligible [15]. When it is possible to check for it, for eukaryotic pathogens, using loci that are located on different chromosomes is recommended.

Particularly striking evidence for LD is the parallel evolution of different parts of the genome, which is the basis of the *g* test for LD [88]. This can be detected by (i) a statistical correlation (measured by a nonparametric Mantel test [45]) between genetic distances obtained from different markers, and (ii) congruence between phylogenetic trees obtained from different multilocus markers (see Fig. 20.4: an extreme example in *T. cruzi*) or from the sequence of different genes [24,25]. Conversely, lack of congruence between trees obtained with different genetic markers is an indication for genetic recombination/sexuality. As mentioned earlier, here a phylogenetic approach (see below) is used as a test for a population genetics problem.

Recently, more refined approaches have been made available [6]. It is encouraging to note that the results they provide closely follow those of more traditional approaches: *T. cruzi* still appears as much more clonal than does *P. falciparum*.

20.4.2.1.2 Artifactual LD: the Wahlund effect LD shows obstacles to genetic recombination, whatever its cause. Trivial, physical obstacles (space and/or time) could therefore generate artifactual LD (the Wahlund effect [35]). It is therefore important to distinguish LD caused by in-built biological properties (clonal evolution, cryptic speciation) from LD simply originating from physical obstacles. A sampling strategy is essential to avoid this bias. To discard the Wahlund effect, only populations collected in sympatric conditions over short spans of time should be analyzed together.

20.4.2.2 Phylogenetic analysis The precise goal of phylogenetic analysis is to show to what extent (i) present species and groups of species correspond to clades and (ii) these species may be additionally subdivided into lower clades. Phylogenies reconstruct the evolutionary past of a species, whereas population genetics give a picture of its present genetic diversity and level of population differentiation. The time scales addressed by the two approaches are therefore not identical. However, in some cases this distinction is more didactic than real (see Fig. 20.4 and point (3) below).

Many different free computer programs are available to compute phylogenetic analyses (for an excellent recent compilation, see [31]). The theoretical principles of each approach should be kept in mind, as the various approaches are not equivalent, a point that is neglected by many empirical users of such software.

The relevant specialized terms dealing with phylogenetic analysis can be found in the Glossary. Detailed information

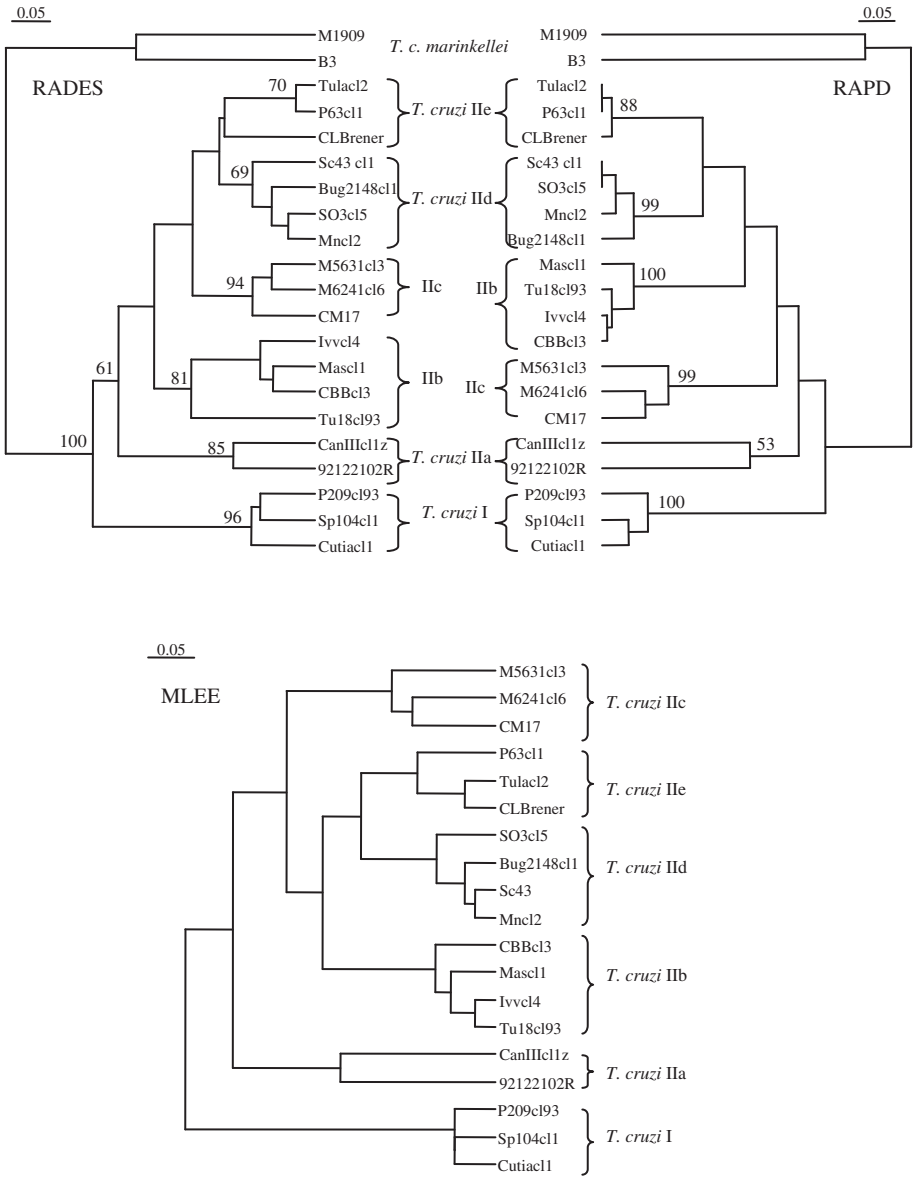

Fig. 20.4. Three phylogenetic trees designed for the same set of stocks of *T. cruzi*, the parasitic protozoan responsible for Chagas disease. Top left: variability of expressed genes surveyed by the RADES technique. Top right: RAPD. Bottom: MLEE. The genomic regions explored by these three kinds of markers are quite different. However, branching of the three trees is very congruent. This shows that these regions do not evolve independently from each other. By definition, this is a manifestation of a strong LD among them, which is an additional confirmation that this parasite undergoes predominant clonal evolution (after [70]; see also Fig. 20.3).

on the various procedures and theoretical implications of phylogenetic analysis are communicated in Chapter 17. A few important principles will simply be recalled here:

1. One should keep in mind the indispensable distinction between *gene trees* and *species trees* [52]. In the same species, different genes may have very distinct evolutionary patterns. Phylogenies based on only one gene or a limited set of genes may not be a reliable reflection of the overall phylogeny of the whole species. As an example, we have seen in Chapter 19 the strange story of the ancient coalescence of some HLA alleles in the human species, resulting in some human alleles being genetically closer to some *Macaccus* alleles than to other human alleles, due to balanced selection of infectious diseases. With this in mind, it is obvious that a phylogeny of anthropoids based on these genes would be aberrant. It is a phylogeny of the molecular evolution of these specific genes, not of the entire anthropoid group. A diversified sample of genes is required to design reliable *species*

trees. It is quite possible that phylogenies based on classical multilocus markers such as MLEE or RAPD are more reliable than gene phylogenies based on only a few genes.

2. Considering the goal of ME, lower levels of evolutionary divergence (microevolution) are the most informative: molecular phylogeny should not be indispensable to distinguish between *E. coli* and *Toxoplasma gondii*! It should be used to distinguish between *Leishmania guyanensis* and *Leishmania braziliensis*, two very closely related species of *Leishmania* in South America, which are morphologically indistinguishable from each other and whose geographical range overlaps.
3. Even with species that are predominantly clonal, such as *T. cruzi*, the agent of Chagas disease [88], some genetic exchange is always present in natural populations, which clouds phylogenies [87]. Actually, as already noted, phylogenetic analysis may be used as an indicator of genetic exchange, to the extent that it produces "bad" phylogenies, with discrepancies between phylogenies obtained from different markers or genes and/or low bootstrap values. Because some genetic exchange is observed in all pathogen species and indeed some species' subdivisions originate from hybridization events [28], *the term "clade" is often highly inadequate for pathogens*, which led to designing the discrete typing unit (DTU) concept [78,87] (Box 20.3).

BOX 20.3: OPERATIONAL CONCEPTS: CLONET, DISCRETE TYPING UNITS (DTUS), TAGS, AND CLONETS [78,87]

Medical professionals need simple, clear-cut answers that are not always compatible with the strict criteria of evolutionary concepts. To fill this gap, we have forged the following descriptive concepts that are easy to understand for non-evolutionists, bypassing certain demands of classical evolutionary biology definitions.

DTUs and Tags

Very often (if not always), the genetic subdivisions identified by evolutionary studies in pathogen species do not fulfill the criteria demanded by a rigorous cladistic analysis. The main reason is that, even in predominantly clonal species such as *T. cruzi* [88], there is some level of genetic recombination that clouds the discreteness of phylogenetic subdivisions. Moreover, some pathogen lineages may have undergone hybridization events [28], in other words, they may have two ancestors. The clade concept is not operational in this case.

However, even when some genetic exchange occurs, discrete and stable subdivisions can be reliably delimited in many cases. We have proposed the term DTUs, sets of stocks that are genetically closer to each other than to any other stock, and are identifiable by common molecular, genetic, biochemical, or immunological markers called tags. DTUs correspond to reliable units of analysis for applied studies in which the genetic diversity of the pathogen has to be taken into account (downstream studies), an ideal target for ME.

Clonet

One of the main goals of ME is to identify clones, that is to say, sets of stocks that are genetically identical and are therefore assumed to be the result of a recent common clonal ancestry. However, because the resolution power of any marker is limited, such sets of supposedly identical genotypes are at best families of closely related clones. The term "clonet" has been coined to refer to those sets of stocks that appear to be identical with a given set of genetic markers *in a basically clonal species*. The clonets are relevant units of analysis for ME. For example, a MLST sequence type (see Box 20.1) or a PFGE pulse type can be equated to a clonet, if the species considered is basically clonal. However, it is crucial to keep in mind that the most recent common ancestor of a given clonet can be either a few weeks or hundreds of years old, depending on the power of resolution (conditioned by its molecular clock) of the markers used. This parameter should be considered for every category of marker, because it cannot be inferred *a priori*. The epidemiological implications of the age of this common ancestor are obviously considerable.

4. As already emphasized, dendrogram clusters designed with any of the variety of software available on the market (see Chapter 17 and [31]) should not be necessarily considered as independent evolutionary units. Computers always generate dendrograms, sometimes with high bootstrap values, even when human populations are concerned (see Figs. 19.2 and 19.4).

20.4.3 Pathogen Species, Subspecies, Strains, and Clones

Unexpectedly for nonspecialists, the terms listed above have no consensual definition among scientists. It is therefore indispensable to try and clarify them, because the basic task of ME is to identify these categories.

20.4.3.1 What good is classifying organisms? First of all, the usefulness of classifying the living world into categories should be clarified, which is by no means obvious. Even many biologists have doubts and consider taxonomists

(specialists of animal/plant classification) as old-fashioned, dusty obsessed, picturesque collectors of butterflies. However, the same skeptics are highly relieved when they have been hospitalized with flu symptoms and learn that the strain is not H5N1 (the virus that causes avian flu). Classifying a virus as H5N1/non-H5N1 *is* taxonomy. The need for classification is indisputable. Scientists, like most people, need to communicate with their colleagues and to identify the things they speak about using a reliable international system.

20.4.3.2 Some basic rules of classification Classification of the living world is based on the hierarchical COFGS system from the broadest to the smallest category: class, order, family, genus, species. Among these categories, the only one that has been the object of attempts to give it a precise biological definition is the species (see below). The other categories are defined by their hierarchical rank, like a set of Russian nesting dolls: there are several orders within a class, and so on. The species, whatever its biological definition (there are many of them), is given a double name in Latin, traditionally written in italics. First, the name of its genus, then the name of the species itself. For example, we refer to the species *Homo sapiens* (genus *Homo,* species *sapiens* [which means wise, possibly an unfortunate choice], the only species currently in the genus *Homo*). *H. sapiens* is a mammal (class), order primates, family Hominidae. Latin names are indispensable for scientists of different countries to communicate. The few rules recalled here are very strict and are accepted by all scientists within the framework of international codes.

20.3.4.3 Artificial versus natural classifications The first goal of classifications is to give names to categories that are agreed on by all scientists. A second, more ambitious step is to build natural categories, in other words categories that are defined according to inferred evolutionary relationships. With this view, mammals are considered as a group of organisms that are more closely related to each other than to any other category (such as reptiles or birds). Within the mammals, primates are more related to each other than to felines or ruminants, and so on.

20.3.4.4 The brainteaser of the species concept In the modern theory of evolution, the species is considered as the basic evolutionary unit. For this reason, great effort has gone into giving this category a precise biological meaning. One has to acknowledge that no agreement has been reached among biologists. Box 20.4 gives a list of the main species concepts currently in use.

BOX 20.4: DEFINITION OF DIFFERENT SPECIES CONCEPTS

Agamospecies: Species that has no regular and/or obligatory biparental mode of reproduction and therefore cannot meet the criteria for biological species. Most pathogen species fall into this category, as are many self-fertilizing and parthenogenetic higher eukaryotes (see Fig. 20.5).
Biological species [23]: "Species are systems of populations: the gene exchange between these systems is limited or prevented by a reproductive isolating mechanism or perhaps by a combination of several such mechanisms."
Cohesion species concept [71]: The most inclusive population of individuals having the potential for cohesion through intrinsic cohesion mechanisms.
Cryptic species: Synonymous with sibling species.
Evolutionary species concept [65]: A lineage (ancestral–descendant sequence of populations) evolving separately from others and with its own unitary evolutionary role and tendencies.
Genospecies [32]: In bacteria, a group of strains with more than 70% sequence homology, estimated by DNA/DNA reassociation.
Phenetic species: A species defined by a phenetic/numerical taxonomy approach [66]: its members share a high number of common traits. Should not be confused with phenotypic species.
Phenotypic species: Species defined of phenotypic traits that can be very few or unique (e.g., pathogenicity).
Phylogenetic species [18]: A monophyletic group composed of the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent.
Sibling species: Biological species that cannot be distinguished from each other by morphological traits.
Species genome concept [43]: Pathogen species are delimited by multilocus sequence typing (see Box 20.1). This concept relies on the fact that bacterial species have two categories of genes: those genes that are shared by most strains of a given species (core genes) and those genes that are not (dispensable genes).

Fig. 20.5. This beautiful insect, *Saga pedo,* from Southern Europe, although quite sexy, is asexual (clonal).

The most popular species concept is undoubtedly the biological species concept, also called the mixiological species concept. Chimpanzees and humans are different species because they are unable in the present state of science to achieve fertile offspring. Horses and donkeys are also different species because even if they produce offspring (mules), they are sterile. There is therefore no gene flow between horses and donkeys, which is the main point from an evolutionary point of view. Indeed, they are forever independent evolutionary units whose fate is to diverge more and more.

The species concepts are used either alone or in cocktails. One species can correspond to several species concepts at the same time. For example, *H. sapiens* is at the same time a biological species (because all humans can have fertile offspring together and cannot with chimpanzees), a phylogenetic species (because all humans are assumed to be closely related from an evolutionary point of view), and a phenotypic species (because all humans have many phenotypic traits in common, whatever the surprising phenotypic plasticity of humans (see Chapter 19). However, they cannot be considered an agamospecies because this category is reserved for those unfortunate organisms that do not enjoy sex.

In spite of the extreme view of some evolutionists, who claim that they are good for birds only [21], biological species do exist in many kinds of pluricellular organisms. They are the fact of recurrent, obvious observations. Any reasonably trained naturalist knows that there are biological species in insects, mammals, birds, reptiles, amphibians, and so on. An interesting specific category of biological species is sibling species, those species that are genetically isolated but are morphologically identical. This is a very frequent case in vectors of infectious diseases. For example, one of the main vectors of malaria in Africa, *Anopheles gambiae*, is actually a complex of six sibling species whose geographical distribution, ecological preferences, and vectorial role are not identical. For malaria control, it has therefore been quite relevant to be able to distinguish all six of these sibling species.

When pathogenic microorganisms are considered, species definition is even more a brainteaser for two reasons: (i) for closely related species (e.g., members of the genus Leishmania, parasitic protozoa responsible for leishmanioses; see Chapter 6), morphological identification is impossible. All Leishmania species (there are many of them) are very similar under the microscope. (ii) Many micropathogens undergo predominant clonal evolution [88] (see below). In other words, they have little or no sex. Because the biological species concept [23] is based on sexual isolation, this species concept is not applicable for these clonal micropathogens.

20.4.3.5 The phenotypic species concept in pathogens: the importance of medical criteria Many micropathogens of medical interest have been described according to medical criteria (clinical or epidemiological features). This makes them *phenotypic species* (see Box 20.4), as the notion of phenotype includes not only morphological traits but also all observable characteristics. Examples include *Leishmania infantum*, the agent of infant leishmaniasis (see Chapter 6), *L. braziliensis*, the agent of mucocutaneous leishmaniasis in Brazil and other Latin American countries, *Vibrio cholerae,* the agent of cholera, and so on. Medical criteria are of course essential to identifying pathogens, as the first goal of medical doctors is to reliably characterize those pathogens that are harmful. However, one has to keep in mind that medical properties are often driven by a limited number of genes. If one intends to build a natural classification of pathogens (see above), that is, a classification that reflects their overall evolutionary relationships, there is a high risk of a strong discrepancy between medically based and natural classifications. An extreme case is the bacterial genus *Shigella,* which cause severe dysentery. Medical doctors have been so struck at the remarkable pathogenic properties of these microbes that they have set it up as an independent genus divided into several distinct species. When *Shigella* isolates are analyzed with evolutionary genetics tools, they are definitely included in the species *E. coli*, the harmless bacterium that colonizes everyone's gut. From an evolutionary point of view, Shigella are simply a set of virulent *E. coli* mutants. Now when you harbor a *Shigella,* you clearly understand the difference with a "normal" *E. coli*.

20.4.3.6 Trouble with the phylogenetic species concept in pathogens The phylogenetic species concept [18] (see Box 20.4) states that a species corresponds to a monophyletic line, evolutionarily independent from other species. It is an attractive concept, because it is clear-cut. It is tending toward becoming a universal concept (the phylocode; http://www.ohiou.edu/phylocode/).

Where micropathogens are concerned, this concept is widely used in different forms that do not always fulfill the rigorous requirements of cladistics. One can cite (i) those categories that are delimited by the use of multilocus sequence typing (Box 20.1): genomic species, the species genome concept [43], and ecotypes [16] (see below); and (ii) for genospecies (Box 20.4).

The phylogenetic species concept is often difficult to apply strictly to pathogens [84]. Indeed, many pathogen species are characterized by a double mode of reproduction. Clonal evolution and sexual reproduction are both used. In the case of many bacteria, exchange of genetic material (broad-sense sexuality) is possible even between distantly related species. Whatever its precise cytological modalities, sexuality has similar consequences. It lowers LD, shuffles genotypes, clouds phylogenetic individualization, and counters phylogenetic divergence. If some sex occurs between different evolutionary lines, the strict criteria of the phylogenetic species concept are impossible to apply (see Box 20.3).

Another possible problem with the phylogenetic species concept is the way it is applied: should one be satisfied when discrete evolutionary lines have been drawn, or should these independent lines differ from each other *by a given level of evolutionary divergence*? In this case, what is the acceptable level of divergence below which it is the same species and above which it is wiser to identify different species? Bacteriologists have tried to settle the problem with the genospecies concept

(Box 20.4). Of course, deciding on a fixed level is somewhat arbitrary. It can be noted that the strategy adopted can differ between specialists.

20.4.3.7 Need for a pragmatic attitude As often occurs when describing the living world, concepts that are too strict are difficult to apply. "Nature makes light of our classifications." However, as underlined at the start of this chapter, these are practical problems, not only basic science. Medical doctors, veterinarians, agronomical engineers, and decision makers are not ready to hear that the species concept in bacteria and parasites is too complex and that we have to abandon describing species. Such an extreme attitude [21] leads to a divorce between evolutionists, on the one hand, and field researchers, health professionals, and so on, on the other [84]. It is all the more desirable to adopt a pragmatic approach, as species in bacteria and parasites do exist. At least this chapter's author adheres firmly to this view. Whatever their evolutionary mechanisms of appearance, stable evolutionary units in pathogenic microbes are corroborated by recurrent, obvious observations. They may not be strictly discrete. This is not a reason for giving up characterizing them. A lack of strict boundaries is a general law of the living world, of the world, period. Are there strict boundaries between adolescence and adulthood? Between day and night? No. However, these entities do exist and are distinct. I am therefore stating that in the world of pathogens, one can reliably observe *and characterize* clear *discontinuities*, stable evolutionary units that deserve to be given the name of species. The evolutionary mechanisms that are at the origin of these species and that keep them stable are still hypothetical. However, the problem does not lie in the very existence of species, but in the difficulties in clarifying the evolutionary mechanisms of their origin and maintenance. Difficulties explaining a biological phenomenon cannot be considered evidence that this phenomenon does not exist. These stable evolutionary units can be characterized using phylogenetic criteria, provided that they are used in a flexible and pragmatic manner. If too strictly applied, the almost constant presence of genetic exchange between evolutionary lines of pathogens will lead to misleading conclusions.

A committee of specialists has recently issued a pragmatic proposal for a polyphasic definition of species in bacteria that could be applicable to other pathogens: "A category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions" [61]. The notion of a high degree of similarity for many characters is a phenetic approach of the concept of species (see Box 4), whereas the idea of genomic coherence is a loose phylogenetic criterion. The authors are modest enough to present their approach as "arbitrary and artificial, but operational." Species described according to these criteria most probably will correspond to real evolutionary units.

Newly described species of pathogens should be relevant for doctors, veterinarians, and agronomy specialists. Applying the above-described criteria could lead to the confusing description of many species. It is obvious that the world of pathogens is discontinuous [84]; however, there are many discontinuities. It is therefore desirable to reserve the honor of new Latin names to those newly identified entities that have a clear usefulness for applied science.

Hardly a joke: the best species concept?

"A species is what a wise and competent specialist thinks to be a species".

20.4.3.8 New concepts: ecotypes and clones "that deserve a name" The ecotype approach [16] has not been proposed to dethrone the presently described species, but more modestly to identify meaningful subdivisions within them. This concept was designed because the traditional species (e.g., *E. coli*) are overly broad units that occupy many different ecological niches and therefore do not correspond to coherent evolutionary lineages. The idea is that only those strains that share the same ecological niche enter into competition with each other, whereas strains pertaining to different ecological niches do not.

The notion that certain clones should be named [43] comes from the observation that some pathogenic bacterial species seem to correspond to recent clonal lines. This is the case for the agent of plague, *Yersinia pestis*. It is genetically extremely homogeneous and can be equated with a recent clone. Giving a specific name to these epidemiologically relevant clones has therefore been proposed: *Y. pestis* would become *Y. pseudotuberculosis* clone Pestis. The difficulty here is to reliably define what a clone is [87] (see Glossary, Section 20.4.3.10, and the clonet concept, Box 20.3).

20.4.3.9 Are there subspecies in pathogens? The concept of subspecies (or geographical race) is discussed at length in Chapter 19. This is not deserved in the case of pathogens because the classical subspecies concept (morphologically dissimilar geographical populations of the same biological species) is not usable. However, pathogen subspecies are described and receive trinames such as so-called classical subspecies. For example, *Trypanosoma brucei*, the agent of human African trypanosomiasis (HAT), is traditionally subdivided into three subspecies, *T. brucei gambiense*, the agent of HAT in West Africa, *T. brucei rhodesiense*, the agent of the East African form of the disease, and *T. brucei brucei*, which is a cattle parasite. These entities could have been named species as well. This is really a matter of appreciation. It can be said that specialists describe microbe subspecies when they dare not describe a new species, by using exactly the same criteria, either phenotypic or phylogenetic, or phenetic, or mixtures thereof. It is probable that the notion of subspecies has little relevance with pathogens.

20.4.3.10 "Strain": a dangerous term At a meeting organized a few years ago on ME, I presided a roundtable entirely devoted to the definition of the term "strain." The number of different definitions roughly equalled the number of participants. Intuitively, the term, in laboratory jargon,

refers to a given collection of pathogen cells you have in a culture tube or box on your bench or in your incubator. The correct term here should be "stock".

A stricter definition of "strain" was proposed by WHO years ago. A strain is a set of stocks from the same species that share given properties identified by the observer, for example, a certain level of pathogenicity, or of antibiotic resistance.

The more operational definition of strain is "a specific multilocus genotype" of a given species. It has the appearance of simplicity; however, it opens the door to new problems, including how to characterize multilocus genotypes (MLGs) and the problem of MLG stability in space and time, which already has been discussed in Section 20.4.2.1.1., and will be further discussed below.

20.4.3.11 The mysterious world of uniparental (clonal) organisms The definition of the term "clone" is touchy. The definition used in the present chapter is genetically based (all cases where the offspring is genetically identical to the parent) and has no reference to the particular mating system of the organisms under study. Many different mating behaviors can produce genetic clones, even in higher organisms. In gynogenetic fishes, the female mates with a male of a related species. However, the spermatozoid serves only to stimulate the division of the ovule. Its genetic material does not penetrate the female gamete. The offspring is a clone of daughters that are genetically identical to their mother [59]. Many insects are parthenogenetic. For example, the magnificent and rare grasshopper *Saga pedo* from Southern Europe (Fig. 10.5) does not even mate. The male is unknown. The mother gives birth to daughters that are genetically identical to her and to each other.

In the perspective of ME, it should be recalled that clonal propagation and clonal evolution are omnipresent in the life of most pathogens [83,88], although genetic recombination (broad-sense sex) also plays a role in most species. This results in clones of pathogens spreading in nature [41]; a basic approach of ME is to identify, characterize, and follow them. Long-term characterization over years and continents is possible, because in those species that are predominantly clonal, *the clones* (*MLG*) *are extremely stable in space and time*. Indeed, their genotypes are not shuffled by genetic recombination. The importance of this notion was recognized very early (see the clone concept in bacteria [55]). Now each time a clonal genotype undergoes any mutation, it splits into two clonal genotypes. With highly mutating sequences, this happens extremely frequently; therefore, it is difficult to settle on an adequate resolution level to identify clones, and the relevance of the clonet concept (Box 3).

20.5 CONCLUSION

As technology progresses under the selective pressure of the market and of scientific competition, the cost of sophisticated molecular techniques will lower more and more, which will make them more accessible to many laboratories, including in developing countries (Chapter 35). This is desirable, as the tools presently available, although already powerful, could be vastly improved. However, it would be misleading to consider that the only necessary progress is in the domain of technology. We have seen in Section 20.4 that many parts of pathogen evolutionary biology remain obscure. It is more than ever necessary to *develop conceptual research* before ME becomes a routine approach [80]. This effort will of course be supported by the parallel and synergistic progress of megatechnologies.

Basic research on pathogen genetics and evolution should not be conducted in isolation. Pathogen and host evolution strongly influence each other (coevolution). Infectious diseases have been and still are the main selective pressure on humans [33]. In the case of vector-borne diseases, coevolution involves three players. It is therefore desirable that the two or three (vector-borne diseases) pieces of the puzzle are put together *in an integrated approach* [78]. For example, people working on the pathogenicity of pathogens should be aware of the role played by human genetic diversity on the severity of infectious diseases (see Chapter 19).

This concept is at the origin of the Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases (MEEGID) congresses, and of the new scientific journal *Infection, Genetics and Evolution* (http://www.elsevier.nl/locate/meegid).

ABBREVIATIONS

AFLP:	Amplification fragment length polymorphism
AIM:	Ancestry informative marker
BP:	Base pairs in the DNA sequence
ERIC:	Enterobacterial repetitive intergenic consensus sequences
ET:	Electrophoretic type
HapMap:	International Human Haplotype Map (project)
HAT:	Human African trypanosomiasis (formerly sleeping sickness)
HGDP:	Human Genome Diversity Project
HGP:	Human Genome Project
HLA:	Human leukocyte antigens
IS:	Insertion sequence
LD:	Linkage disequilibrium
MLEE:	Multilocus enzyme electrophoresis
MLG:	Multilocus genotype
MLST:	Multilocus sequence typing (see Box 1)
PFGE:	Pulse field gel electrophoresis
PCR:	Polymerase chain reaction
RADES:	Random amplified differentially expressed sequences
RAPD:	Random primed amplified polymorphic DNA
REP:	Repetitive extragenic palindromic elements
RFLP:	Restriction fragment length polymorphism
SNP:	Single-nucleotide polymorphism
WHO:	World Health Organization

GLOSSARY

Allele: Different molecular forms of the same gene.

Allelic frequency: The ratio of the number of a given allele to the total number of alleles in the population under survey.

Amplification fragment length polymorphism (*AFLP*): Selective amplification of genomic restriction fragments (obtained by RFLP) by PCR.

Apomorphic: *cf*. cladistic analysis.

Balanced polymorphism/balanced selection: Genetic polymorphism that persists in a population because the heterozygotes for the alleles concerned have a higher fitness than either homozygote.

Bioinformatics: Use of computing technology to process biological data. Often used to refer either to computational molecular biology (the use of computers to analyze the structure and function of genes) or to computational molecular phylogenetic analysis.

Bootstrap analysis: In phylogenetic analysis, generation of pseudoreplicate data sets by randomly sampling the original character matrix to create new matrices that are the same size as the original. The frequency with which a given branch is reproduced by this randomization procedure is recorded as the bootstrap proportion. These proportions can be used as a measure of the robustness of individual branches in the tree. A bootstrap value of 95 for a given branch means that this branch has been found 95 times out of 100 by the procedure.

cDNA or copy DNA: A DNA strand copied from messenger RNA with reverse transcriptase. cDNA therefore corresponds to an expressed gene.

Clade: Evolutionary lineage defined by cladistic analysis. A clade is monophyletic (it has only one ancestor) and is genetically isolated (which means that it evolves independently) from other clades.

Cladistic analysis: A specific method of phylogenetic analysis based on the polarization of characters that are separated into ancestral (plesiomorphic) and derived (apomorphic) characters. Only those apomorphic characters common to all members of a given clade (synapomorphic character) are considered to have a phylogenetic value. For example, feathers are specific of the clade "birds" and are featured by all birds. They are a synapomorphic characters of that clade.

Clone, clonal, clonality: From a genetic point of view, this term designates all cases in which the daughter cells are genetically indistinguishable from the parental cell, whatever the actual mating system.

Coalescence time: Time elapsed between the common ancestral copy (one gene in one individual) and two or more copies of a given gene at the present time.

Crossing over: Reciprocal exchange between two homologous chromosomes during meiosis. Leads to allele exchange and genetic recombination.

Dendrogram: A branching diagram, sometimes resembling a tree, that provides one way of viewing genetic data to suggest similarities and differences between groups or samples.

Ecotype: Population of organisms occupying the same ecological niche, whose divergence is purged recurrently by natural selection.

Electrophoretic type (ET): Set of stocks that have identical MLEE profiles. More currently used for bacteria. CF zymodeme.

Gene: A DNA sequence coding for a given polypeptide. More broadly: any given DNA sequence.

Gene sequence (or genetic sequence or DNA sequence): Can be compared to a series of letters representing the primary structure of a real or hypothetical DNA molecule or strand. The possible letters are A, C, G, and T, which correspond to the four nucleotide subunits of a DNA strand (adenine, cytosine, guanine, and thymine). This coded sequence represents basic genetic information. A DNA sequence may code for proteins. In this case, it directly monitors the succession of amino acids that constitute the primary structure of the protein. Some other sequences have no known coding function.

Genetic distance: Various statistical measures inferred from genetic data, estimating the genetic dissimilarities among individuals or populations. Genetic distances can be based on percentage of band mismatches on gels (the cases for markers such as MLEE or RAPD) or allelic frequency differences or percentage of sequence divergence.

Genetic drift: Random fluctuations of allelic frequencies over time.

Genomics: Molecular analysis of the whole genome of species.

Genotype: Genetic constitution of a given organism; *cf*. phenotype.

Geographical race: *cf*. subspecies.

Homoplasy: Possession in common by distinct phylogenetic lineages of identical characters that do not originate from common ancestry. The origin of homoplastic characters include the following: (a) convergence (possession of identical characters derived from different ancestral characters, due to convergent evolutionary pressure, for example, the wings of birds on one hand and of bats on the other hand); (b) parallelism (possession of identical characters derived from a single ancestral character, and generated independently in different phylogenetic lineages); and (c) reversion (restoration of an ancestral character from a derived character).

Human leukocyte antigens (*HLA*): Cell surface proteins detected by blood testing that exhibit considerable diversity among

individuals. Also called histocompatibility antigens or tissue antigens because organ recipients and donors should have compatible HLA genotypes; otherwise, the transplanted organ is recognized as nonself (foreign) and is rejected. This set of genes is involved in the presentation of antigenic peptides to the immune system and plays a major role not only in tissue compatibility but also in infectious processes.

Insertion sequence (*IS*): A small bacterial transposable element, approximately 1000 bases long, with short inverted repeated sequences at its ends.

Isoenzymes: Protein extracts of given biological samples are separated by electrophoresis. The gel is then processed with a histochemical reaction involving the specific substrate of a given enzyme. This enzyme's zone of activity is then specifically stained on the gel. From one sample to another, migration differences can appear for this same enzyme (see Figs. 20.1 and 19.3, Chapter 19). These different electrophoretic forms of a single enzyme are referred to as isoenzymes or isozymes. When given isoenzymes are driven by different alleles of a single gene, they are more specifically referred to as alloenzymes or allozymes. Differences in migration result from different overall electrical charges between isoenzymes. Overall electric charges are a resultant of the individual electric charges of each amino acid (AA) of a given enzyme. The AA sequence is the direct result of the DNA sequence of the gene that codes for this enzyme. It is therefore considered (and verified) that isoenzyme polymorphism is a faithful reflection of the genetic polymorphism of the organism under study.

Isolate: A given collection of pathogens isolated at a given time from a given host or vector; *cf*. stock.

Linkage disequilibrium: Nonrandom association of genotypes occurring at different loci.

Locus: Physical location of a given gene on the chromosome. By extension, in the genetic jargon: the gene itself.

Microsatellite: A short DNA sequence of DNA, usually 1–4-bp long, that is repeated together in a row along the DNA molecule. In humans (Chapter 19), there is great variation from person to person (forensic use of individual identification) and among different populations in the number of repeats. Numbers of repeats for a given locus define microsatellite alleles. There are hundreds of places in human DNA and in most other species that contain microsatellites. Microsatellites are fast-evolving markers, with a high resolution level and are found in many other organisms, including in pathogens (widely used in molecular epidemiology).

Molecular clock: In its strict, original sense (more correctly called the DNA clock hypothesis), the concept is that that the rate of nucleotide substitutions in DNA remains constant. In a broader sense, simply how fast the genomic part that codes for the variability of a given marker evolves. This speed is driven by the rate of substitution/mutation. It may be regular or irregular.

Monophyletic: An evolutionary lineage that has only one ancestor.

Monte Carlo simulation: A method that estimates possible results from a set of random variables by simulating a process by computer iterations a high number of times ($\geq 10^3$) and observing the outcomes. In LD tests, the observed genotype frequencies at each locus are used to determine the probability of recording the level of LD actually observed in the data by chance based on panmictic assumptions. If the level of LD is never observed in 10^4 iterations, the level of significance of the LD test is $p < 10^4$, a level that is very frequently observed in clonal pathogens [88] (usually accepted level of significance for a statistical test is $p = 0.05$).

Multilocus enzyme electrophoresis: Isoenzyme analysis based on the analysis of a broad range of enzyme systems. Each enzyme system corresponds to one or several genetic loci. Strains that share the same MLEE profile are referred to as zymodemes (parasitology) or electrophoretic types (bacteriology).

Multilocus genotype: The combined genotype of a given strain or a given individual established with several genetic loci.

Natural selection: Process first described by Charles Darwin that favors certain genotypes to the detriment of others over generations. It is entirely driven by the interaction of an organism with the environment.

Neutral gene neutral polymorphism: A gene/genetic polymorphism that does not undergo natural selection.

Panmixia, *panmictic*: Situation in which gene exchange occurs at random in the population under survey.

Parthenogenesis, *parthenogenetic*: Development of an organism from an egg without fertilization.

Phenotype: All observable characteristics of a given individual or a given population apart from the genome. The phenotype is not limited to morphological characteristics and includes, for example, physiological parameters (blood pressure, etc.) or biochemical ones (level of cholesterol, etc.). The phenotype is produced by the interaction between genotype and the environment.

Phylogenetics: A branch of genetics that aims at reconstructing the evolutionary past and relationships of taxa or of separate evolutionary lines.

Phylogeny: Evolutionary relationships between organisms or genes or molecules.

Polymerase chain reaction (*PCR*): A technique that copies the complementary strands of a target DNA chain through a set of cycles until the needed DNA amount is produced. PCR uses synthesized primers whose nucleotide sequences are complementary to the DNA flanking the target region. The DNA is heated to separate the complementary strands, then cooled to have the primers bind to the flanking sequences. The enzyme Taq DNA polymerase is added and the reaction is left to pass through the required number of replication cycles.

Portable, *portability*: A genetic or molecular marker is portable when the results are indisputably identical for the same set of data in different laboratories, which makes exchange of data easy. Gene sequences are very portable for two reasons: (i) they should be identical for a given gene of a given individual between different laboratories; (ii) results may be simply sent out by email: AATGCCA, etc.

Primer: A short DNA sequence used in PCR, RAPD, RADES, and AFLP experiments that anneals to a single strand of DNA and acts as a starting point to initiate DNA polymerization mediated by the enzyme Taq DNA polymerase. In the usual PCR protocol, the sequences of the primers used are known to be part of the genome of the organism under study. AFLP, RAPD, and RADES protocols rely on primers whose sequence is randomly selected.

Pulse field gel electrophoresis (*PFGE*): Separation of large DNA fragments by a particular electrophoresis technique using alternately pulsed, perpendicularly oriented electrical fields. Strains that share the same PFGE are referred to as pulse types. In the case of bacteria, the large DNA fragments result from the action of a low-frequency cutter (a bacterial endonuclease whose restriction action has a low frequency) on the bacterial chromosome. It is therefore a specific case of RFLP. With parasitic protozoa (Trypanosoma and Leishmania) and yeasts, the large DNA fragments correspond to entire chromosomes (molecular karyotype).

Pulse type: A set of stocks that have identical PFGE profiles. Widely used in bacterial molecular epidemiology [72].

Random amplified differentially expressed sequences (*RADES*) [50]: The messenger RNAs of a given individual are purified and transformed into cDNA by the action of the reverse transcriptase enzyme. Then this cDNA is amplified by a RAPD protocol. The RAPD polymorphism evidenced concerns only expressed genes (at the time and in the conditions of the experiment), whereas the normal RAPD protocol exhibits the polymorphism of all sequences, including those genes that are not expressed, and even noncoding sequences.

Random primer amplified polymorphic DNA (*RAPD*): In the classical polymerase chain reaction (PCR) method, the primers used are known DNA sequences, whereas the RAPD technique relies on primers whose sequence is arbitrarily determined. RAPD primers are generally 10 bp long. The possible combinations are virtually unlimited. For a given genotype of a given individual or strain, different primers will reveal different polymorphisms. RAPDs are an extremely powerful method of exploring the genetic variability of organisms. However, their use in routine strain identification is limited by their lack of reproducibility.

Restriction fragment length polymorphism (*RFLP*): Variability in the DNA of a given organism revealed by the use of restriction endonuclease bacterial enzymes. The endonuclease cuts the DNA at specific restriction sites, and the polymorphism of the DNA fragments thus obtained can be visualized on gels, either directly by ethidium bromide staining or by Southern blot hybridization with specific probes.

Reverse transcriptase: Enzyme that synthesizes a copy DNA (cDNA) from an mRNA template.

Recombination: Reassortment of genotypes occurring at different loci.

Ribotyping: DNA sample under study (isolated from a given strain) is restricted, then hybridized with a probe designed with the ARN 16S ribosomal gene.

Sex, *sexuality*, *sexual reproduction*: In the broad sense, any case of exchange of genetic material between two independent cells (broad-sense sexuality).

Sexual selection: Selection that promotes traits (e.g., the peacock's tail) that increase success in mating.

Sickle-cell anemia: A generally lethal form of hemolytic anemia observed in individuals who are homozygous for the autosomal, codominant gene H^s. The red cells of these individuals contain an abnormal hemoglobin, Hb^s. These red cells exhibit a reversible shape alteration when the oxygen concentration in the plasma falls slightly and develop a sickle-like form. These pathological red cells have a shortened lifetime. Approximately 0.2% of African-American babies suffer from sickle-cell anemia.

Single-nucleotide polymorphism (*SNP*): Polymorphisms or one-letter variations in the DNA sequence. SNPs contribute to differences among individuals and populations. Most of them have no effect, others cause subtle differences in countless features, such as appearance, whereas some affect the risk for certain diseases. Widely used as high-resolution population markers.

Spacer DNA: In eukaryotic and some viral genomes, untranscribed DNA segments that flank functional genetic regions or cistrons.

Stock: A given pathogen line subcultured *in vivo* or *in vitro* starting from a given isolate.

Subspecies (*also called geographical race*): Geographical populations of the same species that exhibit distinct genetically based phenotypic traits making it possible to distinguish most individuals of one population from most individuals of another population.

Sympatry: Living in the same geographical location (*cf.* allopatry).

Synapomorphic: *cf.* cladistics.

Synonymous mutation/silent mutation: Mutation that produces change in DNA but no change in protein due to redundancy of the genetic code.

Zymodeme: Set of stocks that have identical MLEE profiles. More currently used for parasites. *cf.* electrophoretic type (ET).

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CHAPTER 21

The Need for Megatechnologies: Massive Sequencing, Proteomics and Bioinformatics

David G. Biron,[1] Austin L. Hughes,[2] Hugh D. Loxdale,[3] and Hercules Moura[4]

[1]*GEMI, UMR CNRS/IRD 2724, Centre IRD de Montpellier, 911 av. Agropolis, BP 64501, 34394 Montpellier cedex 5, France*

[2]*University of South Carolina, College of Science and Mathematics, Columbia, SC 29208, USA*

[3]*Plant & Invertebrate Ecology Division, Rothamsted Research, Harpenden, Herts., AL5 2JQ, UK*

[4]*National Center for Environmental Health, Centers for Disease Control and Prevention, Division of Laboratory Sciences, MS F-47 Chamblee Campus, BLG 17, Atlanta, GA 30341-3724, USA*

21.1 INTRODUCTION

During the course of human history, infectious diseases have seriously affected many societies worldwide. In Europe, one of the most dramatic disease events was the great plague pandemic of the mid-fourteenth century, which first spread to Italy and Egypt in 1347, probably from northern India via the Crimea, in ships carrying rats infected with fleas (especially the Oriental rat flea, *Xenopsylla cheopsis*) in turn infected with the plague bacillus *Yersinia pestis* [3,126]. In less than 4 years, the plague may have killed as many as 24 million people – around 30% of the European population at the time, there being no remedy for this terrible affliction [126]. From antiquity to the present day, our collective understanding of the causes and mechanisms of the occurrence of infectious diseases has evolved along with progress in scientific thought and knowledge, which has oftentimes, but not always, led to prevention and cure. Only by such rational analysis of the causes and dissemination of infectious diseases can remedies be affected, more especially in the longer term and on a global scale.

At the most basic level, infectious disease results from an intimate relationship between the host organism and the pathogen (or parasite in the case of parasitic organisms), whatever this may be, which involves molecular "cross-talk." Clearly, elucidation of this complex communication between host and pathogen/parasite is desirable, if not essential, in order to improve our understanding of infectious diseases.

During the twentieth century, a newly emerging scientific discipline – molecular biology – was considered by many researchers as the best option to explore and understand such cross-talk. However, by the end of the century, it had become increasingly clear that while it may be "mandatory" to determine a genome sequence apparently involved in cross-talk between host and pathogen/parasite, such determination is not in itself enough to understand complex biological processes, such as immune defenses occurring during biochemical cross-talk events [35,36]. Many parasitologists put their faith in proteomics studies as a means of explaining, as well as vanquishing, many human diseases. Even so, the majority of "parasito-proteomics studies" are as yet still very much dependent upon an extremely simple framework of knowledge. As a consequence, a key question remaining is whether the host genome response seen during a parasite infection represents a nonspecific response that may possibly be induced by any parasite or rather for that matter, any other kinds of stress.

From our present standpoint, three periods can be distinguished with regard to the collective understanding of molecular biological processes, which may be loosely termed "pre-genomic," "genomic," and "post-genomic." The pre-genomic era started with the elucidation of the structure of DNA by James Watson, Francis Crick, and colleagues in 1953 [125], while the genomic era really only began in 1992 with the launching of the Human Genome Project [24]. With the successful complete sequencing of the human genome in

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2003 (and which incidentally was preceded by the genome sequencing of the internationally important malarial pathogen, *Plasmodium falciparum* and its insect vector, the mosquito, *Anopheles gambiae*), the post-genomic era may be said to have started in earnest [21,26,42,51,56,119].

In this chapter, we briefly review the impact of molecular biology on infectious diseases during the pre-genomic and genomic eras. However, our main focus is on the post-genomic era, more precisely the "Eldorado proteomics period and on bioinformatics."

21.2 THE PRE-GENOMIC ERA

Molecular biology may be said to have been established in the 1930s with the coining of the term in 1938 by Warren Weaver, the then director of the natural sciences program at the Rockefeller Foundation in the United States. After the publication of the structure of the DNA double helix on April 25, 1953 by Watson and Crick [124], many researchers developed molecular tools to study DNA, the repository of genetic information, in all the various life kingdoms of life on earth. During the pre-genomic era, many researchers developed and used molecular tools, especially in epidemiology and population genetics, to study the molecular cross-talk in human–pathogen systems, to prevent outbreak of infectious diseases, and more importantly from an applied point of view, to combat infectious pathogens.

21.2.1 Molecular Epidemiology and Infectious Diseases

Outbreaks of infectious diseases have been observed throughout recorded history, and doubtless, long before. Even so, the theories offered to explain how and why outbreaks occur resulted directly from an understanding of the natural forces and risk factors that affected patterns of illness observed in society at the time. Since the discovery of the structure of DNA, molecular tools have been developed in order to detect and identify microorganisms to the strain level, while microbiologists have started using molecular typing to track infectious diseases. Table 21.1 summarizes the molecular tools developed during the pre-genomic era for typing infectious pathogens. Most work on strain typing has focused on bacteria, although fungi, viruses, and other parasite groups have also been typed. Moreover, detection of emerging infectious agents, particularly in certain risk groups such as AIDS patients, has posed many new challenges.

TABLE 21.1. Molecular Tools Developed During the Pre-genomic Era for Typing Infectious Pathogens (Adapted from [116,121,133])

	Common name	Reproducibility	Technical ease	Discriminatory power	Ease of interpretation
Phenotypic methods					
	Biotyping	Poor	Easy	Poor	Moderate
	Serotyping	Good	Easy	Fair	Moderate
Conventional	Antibiotic susceptibility testing	Good	Easy	Poor	Easy
	Phage typing and Bacteriocin typing	Poor	Difficult	Poor	Easy
Molecular	Immunoblotting and electrophoresis protein typing	Good	Moderate	Moderate	Easy
	Multilocus enzyme electrophoresis	Excellent	Moderate	Moderate	Easy
Genomic methods					
Focus on the detection of	AP-PCR	Good	Moderate	Good	Moderate
specific genes	Oligonucleotide probes	Good	Moderate	Good	Moderate
Used the whole genome	Plasmid fingerprinting	Good	Moderate	Good	Moderate
	RFLP typing by probe hybridization	Good	Moderate	Good	Difficult
	REA of plasmid DNA, chromosomal DNA and ribonucleic acid by conventional electrophoresis	Good	Moderate	Good	Difficult
	REA chromosomal DNA with PFGE	Excellent	Moderate	Moderate	Moderate
Specialized	MST	Excellent	Moderate	Excellent	Moderate
techniques	FBAFLP	Excellent	Moderate	Excellent	Moderate
	SNP	Excellent	Moderate	Excellent	Moderate

Note: AP-PCR: arbitrarily primed polymerase chain reaction; FBAFLP: fluorescence-based amplified fragment length polymorphism; MST: multilocus sequences typing; PGFE: pulsed-field gel electrophoresis; REA: restriction endonuclease analysis; RFLP: restriction fragment length polymorphism; SNP: single nucleotide polymorphism.

In any outbreak of an infectious disease, a number of epidemiologic issues need to be addressed in order to improve treatment as well as prevent the spread of the infectious agent in question. For example, it is mandatory to determine whether the isolated pathogen that is causing a particular infection has been transmitted to a healthy person, has been acquired from an environmental source such as a hospital, or if it was a "normal colonizer" of the host that has now become invasive. As for recurrent or persistent infections, the question is whether a single pathogen/parasite strain is causing the infection, or whether a new strain (or strains) with different genotypes has evolved? Strain typing using molecular methods has provided and continues to provide many new insights into several of these issues. A successful example is "PulseNet," where participating laboratories perform standardized molecular subtyping (or "fingerprinting") by pulsed-field gel electrophoresis (PFGE) of food-borne disease-causing bacteria. Originally, a national network of public health and food regulatory agency laboratories in the United States and coordinated by the Centers for Disease Control and Prevention (CDC), PulseNet has expanded and is now being used for international construction of bacterial fingerprinting databases, for the rapid detection of new strains, as well as the spread of pathogenic clones of bacteria through different regions or countries (http://www.cdc.gov/pulsenet/).

21.2.2 Population Genetics of Hosts and/or Infectious Agents

In the pre-genomic era, it was often assumed within the scientific community concerned with infectious diseases that studies on the population genetics of host and/or infectious agent/s, including parasites, would prove instrumental in epidemiological and taxonomic studies. In addition, it was thought that such studies would be valuable for evaluation of genetic diversity of infectious agents with different biological properties such as virulence, resistance to drugs, and immunological diversity. At this time, many molecular tools were developed, more especially for studying the population genetics of organisms from all the various kingdoms of the living world. Of these tools, the most representative developed to study pathogen biodiversity are listed in Table 21.2. More general information on population genetics can be found in a range of reviews [4,10,11,45,75,78, 98,104,118,121].

During the pre-genomic era, studies on the population genetics of pathogens with molecular markers revealed the broad biodiversity of species along with the genotypic variation of many of them. At the same time, it allowed new hypotheses to be developed to explain the emergence of resistant pathogen strains to one or more antibiotics. Furthermore, epidemiologists have been able to exploit genotypic variation in pathogen populations, thereby enabling the discrimination

TABLE 21.2. Summary of Certain Qualitative Characteristics of the Main Molecular Markers Designed and Used During the Pre-Genomic Era for the Population Genetics of Parasites (Adapted from [70,89,91,93,97,100,102,121])

Molecular Techniques (First Reference)	Dominance	Reproducibility[a]	Accuracy[b]	Technical Ease[c]	Cost of Development[d]; Cost by Sample
Allozymes [104]	Codominant	Medium	Medium	High	Low; cheap
SSCP [92]	Codominant	Medium	Medium	Medium	High; expensive
SSR [114]	Dominant	High	Medium	Medium	Intermediate; cheap
AP-PCR [128]	Dominant	Low	Low	High	Low; cheap
RAPD [129]	Dominant	Low	Very low	High	Low; cheap
SCAR [95]	Codominant	Medium	Medium	High	High; reasonable
PCR-RFLP [72]	Codominant	Medium	Medium	Low	Intermediate; reasonable
SAMPL [85]	Dominant	High	High	Low	Low; cheap
ISSR [136]	Dominant	High	High	Low	Low; cheap
AFLP [122]	Dominant	High	High	Medium	Low; cheap
RAHM [22]	Codominant	High	High	Low	Cheap; high
RAMPO [105]	Codominant	High	High	Low	Low; cheap
S-SAP [127]	Dominant	High	High	Medium	Low; expensive
ASAP [19]	Dominant	Low	Very low	Very high	Low; cheap
RAMP [81]	Dominant	High	High	Low	Low; cheap
SNP [123]	Two codominant alleles	High	High	Medium	High; expensive

Note: AFLP: amplified fragment length polymorphism; AP-PCR: arbitrarily primed polymerase chain reaction; ASAP: arbitrary signatures from amplification profiles; ISSR: inter simple sequence repeats; PCR-RFLP: PCR–restriction fragment length polymorphism; RAHM: random amplified hybridization microsatellites; RAMP: randomly amplified microsatellite polymorphism; RAMPO: randomly amplified microsatellite polymorphisms; RAPD: random amplified polymorphic DNA; SAMPL: selective amplification of microsatellite polymorphic loci; SCAR: sequence-characterized amplified region; SNP: single nucleotide polymorphism; S-SAP: sequence-specific amplification polymorphisms; SSCP: single-strand conformation polymorphism; SSR: simple sequence repeats.

[a]Refers to the ability to obtain the same genetic result for the same sample in repeated assays.

[b]Refers to the precision at which true allele recognition can be performed.

[c]Refers to the level of skill required to obtain accurate genetic data once an assay has been developed.

[d]The financial requirements and the time needed to develop genetic assays and procedures for optimal performance.

and tracking of pathogen–parasite species and strains that are responsible for disease outbreaks. Lastly, pathogen–parasite diversity *per se* has proved a rich source from which inferences have usefully been drawn to develop models of the evolution of both pathogen and pathogenicity [50].

21.2.3 Pre-Genomic Era and "Bioterrorism"

Biological and chemical agents have been used as weapons since the early medieval period – from crude forms using human and animal corpses catapulted into castles and fortified towns and cities during sieges to sophisticated preparations developed for use in warfare by national government during and after the Second World War. In more recent times, one report of such bioterrorism by individuals or small groups of individuals was the contamination with *Salmonella* of salad bars in Oregon, United States, in 1984 to prevent people from voting in an election; another, the anthrax attack in the United States that infected 22 people and killed 5 soon after the September 11 ("nine eleven") outrage in New York in 2001 [13,39]. However, during the "pre-genomic" era, a host of molecular diagnostic methods were developed which later became very important in the early detection and diagnosis of intentional use of infectious agents and for recognizing outbreaks of emerging diseases. Now the entire genome sequences of the majority of potential bioterrorism agents is known, with highly sensitive polymerase chain reaction (PCR) gene amplification methods of differing format being applied to detect and characterize these organisms, while PCR has become the preferred method to detect such deadly disease agents as anthrax, plague, tularemia, brucellosis, and smallpox [38].

21.3 GENOMIC ERA

Determination of the complete genome sequence of an organism has captured the imagination of researchers because the information so gained is expected to reveal – reasonably or unreasonably – the "key of life." Thus, the number of genes necessary to make up an organism should, it is hoped, be relatively rapidly known, along with what exactly goes to make up a pathogen, in the case of such agents. Furthermore, information about environmental influences can be gathered through comparative genomics of both host and pathogen–parasite.

21.3.1 Genome Projects

When the technology first appeared in the 1970s and 1980s, the determination of nucleotide sequences using either Sanger's enzymatic method [110] or the Maxam and Gilbert's [82] chemical degradation method was both slow and labor-intensive. Hence, the first pathogens to be analyzed were – not surprisingly (as a direct result of their small genome size) – viruses [37] and bacteriophages, that is, PhiX [109] and T7 [29]. The human mitochondrial genome was another important landmark in the history of nucleotide sequencing and much was learned about the "life cycle" of viruses and their interaction with the infected host cell [7]. As a result of the tedious nature of sequencing, only with the advent of automated sequencing in the late 1980s were ambitious plans first made to launch the Human Genome Project.

Since the start of the genomic era in the early 1990s, and with much faster sequencing technologies now available, the complete genomic sequencing of many microbial pathogens has been quickly achieved, aided again by their small size. This newly acquired body of scientific information has paved the way for an international understanding of genomic features and organization of infectious agents such as bacteria, viruses, and fungi [83,113]. In contrast, however, most parasites have relatively much larger genomes, which makes analysis of gene expression and function in these organisms relatively more difficult [66]. Among the completely sequenced microbial genomes, about 50% belong to agents of infectious diseases in humans. The impetus for sequencing a number of infectious disease pathogens of medical or veterinary importance initially stemmed from researchers within these disciplines from developed countries, and from countries where the disease-causing agents are endemic. A list of web sites concerning various host and pathogen genome projects is shown in Table 21.3.

At present, completion of the sequences of several important host and pathogen genomes offers both a tremendous opportunity as well as a huge challenge. The pre-genomic and genomic eras have been dominated by "reductionist" approaches, the completion of genomic sequences of many organisms being perhaps the single most important achievement of such molecular reductionism (Fig. 21.1a).

21.3.2 New Scientific Fields Emerged During the Genomic Era

Of the scientific disciplines to emerge during the genomic era, "Bioinformatics" has proved especially significant, including in terms of microbial genomic research and involving comparative analysis of genome data from more than one species (see also Section 21.4.2 below). Comparative genomics is an "umbrella" title that contains several different areas of research. Initially confined to gene mapping studies, it has now grown to encompass a range of different aspects, including whole genome analyses that compare relative genome composition and chromosome organization; identification of conserved synteny; characterization of orthologous genes and paralogous gene families, classification of species-specific genes implied in host-specificity and pathogenicity, while it can also be used to chart the evolution of the organisms being thus compared. Although comparison of the whole genome of pathogenic eukaryotes is still in its infancy, significant differences in biochemical pathways between host and parasite have been identified for a number of protozoan parasitic species. At the same time, comparison of the sequenced parasite species-specific gene families identified may subsequently lead to potential drug targets [21,106]. Despite the achievements obtained during the genomic era, it may be too early to predict how quickly emerging genomic data will yield reagents for treatment and prevention of parasitic infectious disease. However, it may be

TABLE 21.3. Selected Web Sources for the Host and Parasite Genome Projects

Subject	Web site
Human genome project	http://www.ncbi.nlm.nih.gov/ http://www.ensembl.org/Homo_sapiens/
Mouse genome project	http://www.nih.gov/science/models/mouse http://www.ensembl.org/Mus_musculus/
Microbial pathogen genomes	http://www-fp.mes.anl.gov/~gaasterland/genomes.html http://www.microbialgenome.org/links.shtml http://www.tigr.org/tdb/mdb/mdbcomplete.html http://www.wehi.edu.au
Parasite genomes	http://www.sanger.ac.uk/projects/ pathogens http://www.tigr.org/tdb/parasites/ http://www.pasteur.fr/recherche/unites/tcruzi/minoprio/genomics/parasites.htm http://www.dbbm.fiocruz.br/genome/genome.html http://www.ebi.ac.uk/parasites/parasite-genome.html
Emerging infectious diseases	http://www.cdc.gov/ncidod/emergplan http://www.cdc.gov/drugresistance/community/ http://www.niaid.nih.gov/dmid/eid/ http://www.earss.rivm.nl http://www.who.int/emc/amr.html

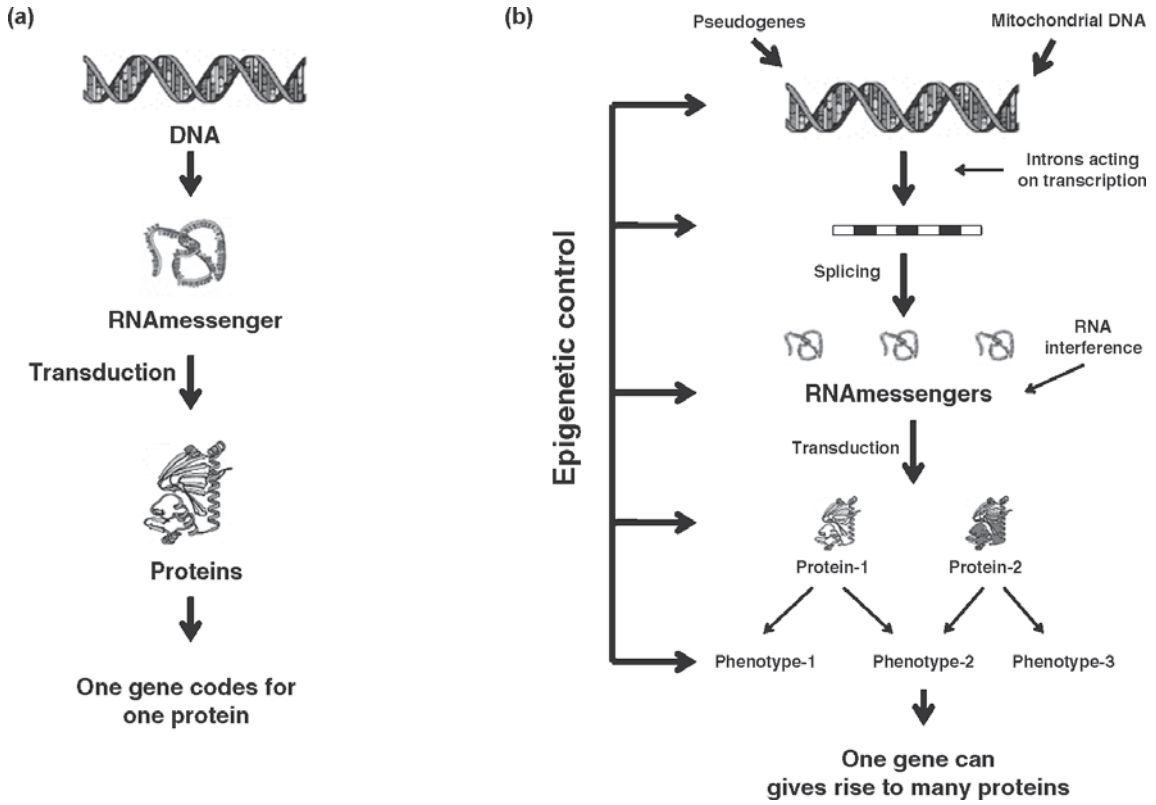

Fig. 21.1. The central dogma of molecular biology of the genomic era (a) and of the post-genomic era (b). See color plates.

predicted that completion of the sequencing of additional host and parasite genomes will surely provide new insights, thereby allowing the development of effective vaccines against a range of pathogenic agents.

21.3.3 Genomic Era and Bioterrorism

Preparedness against both natural and deliberate outbreaks of infectious diseases gained special attention in the genomic era. Based on several criteria, the CDC has classified "critical biological agents" into three major categories – A, B, and C [13] (see also CDC web site, http://www.bt.cdc.gov/agent/agentlist-category.asp#catdef). Category A agents include viruses such as *Variola major*, Filoviruses, and Arenavirus, bacterial agents as *Bacillus anthracis*, *Y. pestis*, and *Francisella tularensis*, and the deadly toxin produced by *Clostridium botulinum*. These agents can be easily transmitted from person to person, have severe (usually fatal) impact on human health, and thus the potential for high mortality, besides being easily developed into weapons. Category B agents include zoonotic bacteria and viruses, food-borne bacteria, and a parasite *Cryptosporidium parvum*, which are capable of producing serious harm and potentially significant mortality to humans, but less so than category A agents. As for category C agents, the list includes mostly emerging pathogens such as viruses and multidrug-resistant tuberculosis bacteria. Sequencing of most of the category A and B agents has now been accomplished. Better understanding on genetic variation of these species and strains is providing important information on the "adaptive plasticity" of the microbial genome and how this impacts upon microbial forensics, epidemiologic studies, the spread of antibiotic resistance and pathogenicity, and the development of new therapeutics and vaccines [39] (see also the chapter by Morse, this volume).

21.4 POST-GENOMIC ERA

Post-genomic research is now firmly established as a major scientific discipline in the twenty-first century and new millennium. Focusing research on individual virulence genes of the most important pathogens has been the traditional strategy to understand and combat human infectious diseases [30,135]. The complexity of the human genome has complicated identification and functional characterization of components of the host defense system against invading microorganisms. There is now an urgent need for global studies using proteomics and bioinformatics to both efficiently and precisely elucidate the complete genome interactions between microbial pathogens and their hosts.

21.4.1 Proteomics

21.4.1.1 Interest to study the host–parasite interactions via their proteomes Until now, molecular biologists have focused their studies on DNA analyses based on the central dogma of molecular biology – that is to say, the general pathway for the expression of genetic information stored in DNA. Thus, DNA is first transcribed into transient mRNAs and decoded on ribosomes with the help of adaptor RNA (tRNAs) to produce proteins, which, in turn, perform all the various enzymatic and structural functions of the whole organism (Fig. 21.1a).

Molecular biologists were confident that complete sequencing of the genome of host–parasite systems would explain most of the infectious and parasitic diseases [26,53]. Unfortunately, despite this expectation, very little progress has been achieved in the control of such diseases, despite decades of intensive genomic projects on host–parasite interactions, vaccines, and chemotherapeutics. Infectious and parasitic agents continue to be a major cause of morbidity and mortality in humans and domestic livestock, more especially in developing countries [43,46,94,108].

As earlier mentioned, by the end of the twentieth century, it had become clear to many molecular biologists that knowing genome sequences, while technically mandatory, was not in itself enough to fully understand complex biological events like the immune defense of a host and/or a parasite during their interaction [9,12,35,36]. The evolution of any given species has tremendously increased complexity at the level of pre-(gene splicing, mRNA editing) and post-translational (phosphorylation, glycosylation, acetylation, etc.) gene–protein interaction. The genomics era has revealed that (i) DNA sequences may be "fundamental," but can provide little information on the dynamic processes within and between host and a parasite during their physical and molecular interaction [9,12]; (ii) the correlation between the expressed "transcriptome" (i.e., total mRNA transcription pattern) and the levels of translated proteins is poor [5,47,79]; (iii) a single gene can produce different protein products [35,36,79]; and (iv) the dogma of molecular biology needs to be revised. In essence, this is because genome, transcriptome, and proteome (i.e., protein complement of the genome or the sum total of cellular proteins) of an organism are mutually interactive [6,53] (see Fig. 21.1b).

The biological phenotype of an organism is not directly related to the genotype because of epigenetic information [130]. Epigenetic systems control and modify gene expression. Almost all the elements of epigenetic control systems are proteins [6]. The cells of an organism are reactive systems in which information flows not only from genes to proteins but in the reverse direction as well [53]. Some authors suggest that the proteome is the genome-operating system by which the cells of an organism react to environmental signals [6]. It comprises an afferent arm, the cytosensorium (i.e., many cellular proteins are sensors, receptors, and information transfer units from environmental signals) and an efferent arm, the cytoeffectorium (i.e., in cells, reaction of the genome via regulation of either individual proteins or a group of proteins in response to environmental changes).

Proteomics (see Box 21.1) offers an excellent way to detect and observe the host genome in action through the evaluation of the host proteome during the host–parasite interactive process (see Fig. 21.3) [15]. Using the first generation proteomics approach – two-dimensional electrophoresis (2-DE)

Box 21.1. WHAT IS PROTEOMICS?

Proteomics is the study of the proteome. In a broad sense, the proteome means all the proteins produced by a cell or tissue. Proteomics is a scientific discipline that promises to bridge the gap between our understanding of genome sequence and cellular behavior; it can be viewed as more of a biological assay or tool for determining gene function. Methodologically, proteomics takes three major forms: proteomics analysis (analytical protein chemistry), expression proteomics (differential display proteomics), and cell-mapping proteomics (cataloging of protein–protein interactions [12, 15].

Proteome studies are traditionally conducted using two-dimensional gel electrophoresis (2-DE) as a separation method and mass spectrometry to identify proteins (see Fig. 21.3) and sophisticated informatics approaches for interrogating data [12,41]. 2-DE is a technique by which several thousand proteins of a species can be separated according their p*I* (isoelectric point) and their mass (Mw). In spite of the capacity of 2-DE to resolve complex mixtures of proteins, it presently has some limitations [12,15]. Hydrophobic proteins, proteins having an extreme p*I* values (<3 and >10) or insoluble proteins are difficult to reveal using with 2-DE. Also, low-abundance proteins may present problems of detection that can be resolved by fractionation or enrichment procedures. One of the most popular mass spectrometric identification methods used in proteomics is MALDI-MS (matrix-assisted laser desorption/ionization-mass spectrometry) (see Fig. 21.2) [12]. MALDI-MS is used to determine the accurate mass of a group of peptides derived from a protein by digestion with a sequence-specific protease, usually trypsin, thus generating a peptide mass map or peptide mass fingerprint (PMF). PMF is, however, not suited to identification of a complex protein mixture. Because trypsin selectively cleaves proteins at basic arginine and lysine, the masses of the tryptic peptides can be predicted theoretically for any entry in a protein sequence database. For given digested protein spots, experimental peptide masses are searched against theoretical fragments from genomic DNA, cDNA, EST, and protein database entries. The quality of the match of the protein to gene is determined first by the degree of agreement between the observed and the theoretical peptide masses which requires a very accurate absolute peptide mass measurement, and secondly, by the proportion of total peptide fragments observed (coverage(%) of protein sequence) [12]. Tandem mass spectroscopy (MS/MS) can be used to generate short amino acid sequences from peptides (see Fig. 21.2). In the first stage of analysis, a peptide mass profile is produced; in the second stage, selected peptide ions are passed into a collision chamber where they interact with a collision gas. This causes fragmentation along the peptide backbone and generates a series of fragments that differ in mass by a single amino acid. The partial amino acid sequences obtained from the MS/MS spectra (the sequence tag) combined with the original peptide masses are then used for database searching in a similar manner to PMF.

and mass spectrometry (MS) – posttranslational modifications of host proteins (such as phosphorylation, glycosylation, acetylation, and methylation) in reaction to parasite invasion can be detected. Such modifications are vital for the correct activity of numerous proteins and are being increasingly recognized as a major mechanism in cellular regulation. Although 2-DE offers a high-quality approach for the study of host and/or parasite proteomes, several proteomic tools have been developed that complement this approach [16,40,47,77,131]. A comparison of the most popular such tools currently in use are shown in Table 21.4.

21.4.1.2 Limitations of the current approach in "parasito-proteomics" Studies in "parasito-proteomics" are performed either by following the expression of the parasite proteome during infection by a given parasite [17,23,74,87], by the reaction of the host proteome following an invasion by a parasite species [23,86,117,125], or by the injection of immune elicitors [49,120] (see Fig. 21.4). However, a key point is to define whether the host genomic responses elicited through activation of immune constitutive proteins – induction and/or suppression of proteins during the infection by a parasite – represent a nonspecific response that might be induced by any pathogen.

The classical approach in parasito-proteomics makes it possible to identify proteins of interest for a given host–parasite system. For example, Wattam and Christensen [125] associated some polypeptides with the genome response of the host mosquito *Aedes aegypti* (Diptera: Culicidae) with the invasion of the filarial worm *Brugia malayi*. This pioneering study provided important new information on the response of the host insect to invasion by a specific parasite species. Nevertheless, it was not possible to determine whether the response detected in *A. aegypti* is specific to *B. malayi* (Spiruria, Filariidae), or whether it can be observed for any parasitic worm species invading a dipterous host.

Recently, Moura and Visvesvara [87] used the classical proteome approach (2-DE /MS) to study protein expression of two human microsporidian isolates, *Encephalitozoon intestinalis* (CDC V:307) and *Brachiola algerae* (CDC V:404) during their multiplication within monkey kidney (E6) cells. The authors initiated the analysis of host–parasite interactions through the

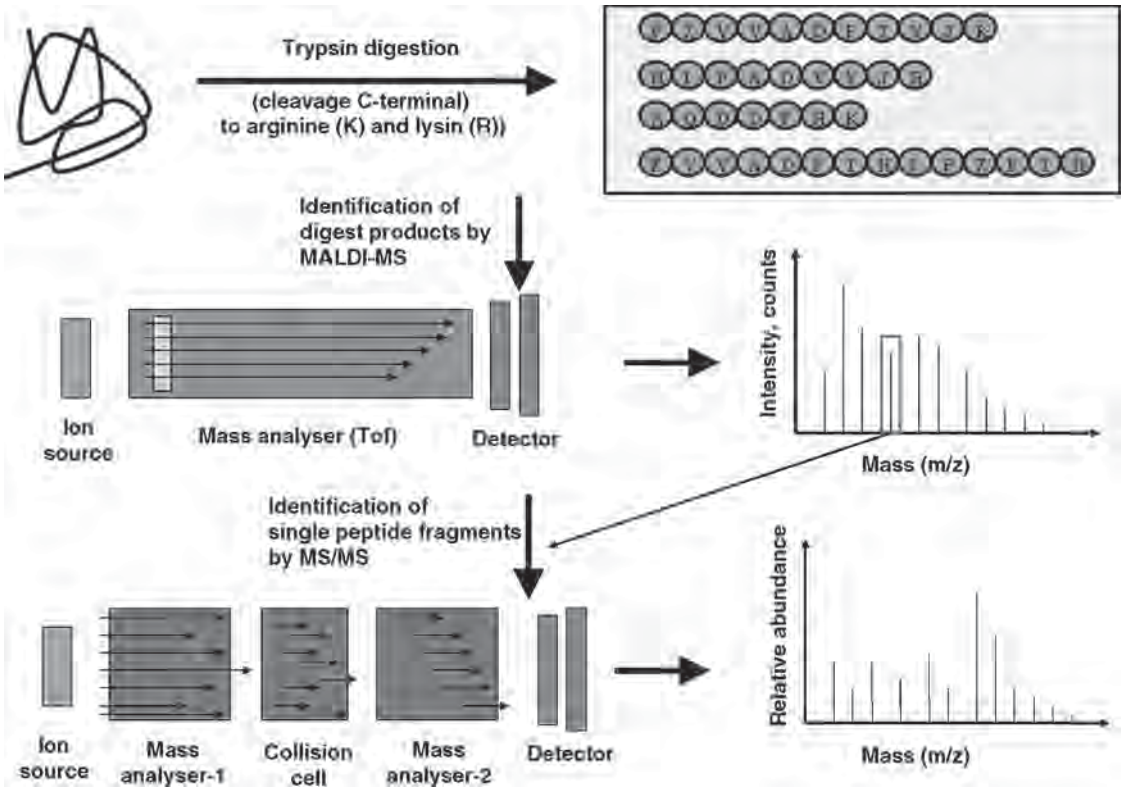

Fig. 21.2. Schematic illustration of the identification of protein by MALDI-TOF MS (mass spectrometry) and by tandem MS. See color plates.

detection of the proteins expressed during infection of E6 cells. When proteins were extracted and separated by 2-DE, approximately 250 protein spots were detected on gels. Spots with molecular masses in the range of <10–200 kDa increased in frequency in the acidic part of the gel. Analysis of gels run revealed proteome changes commensurate with the percentage of cells infected. In addition, immunoblot analysis of gels revealed more than 95 immunogenic spots, including markers for spore maturation, potentially useful markers for both diagnosis and drug targets. These findings agreed with recent data obtained with other organisms using the proteomic approach. For example, immunoblot studies on Lyme's

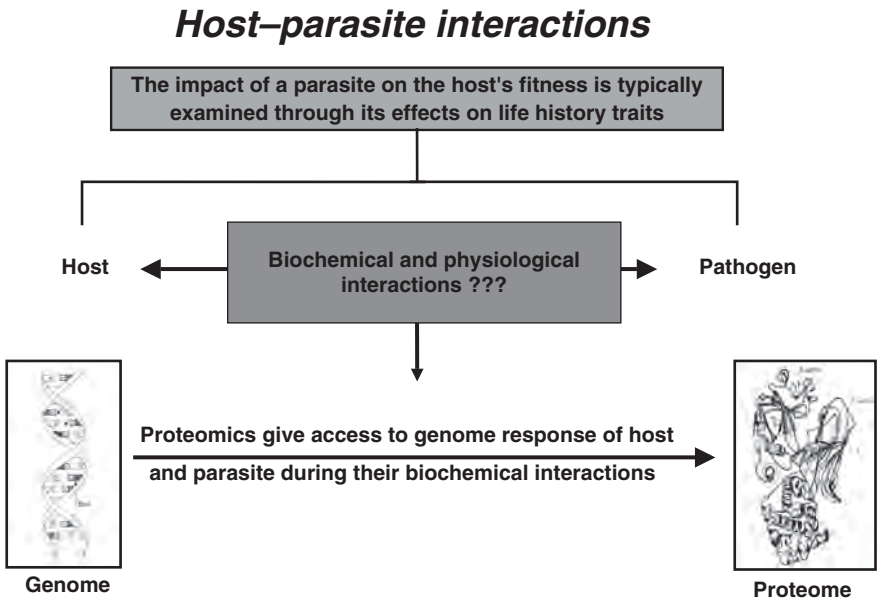

Fig. 21.3. Proteomics, a new methodological approach to study the host–parasite interactions.

TABLE 21.4. Comparison of Different Proteomic Tools

	2-DE	2-DIGE	MuDPIT	ICAT™	SELDI-TOF MS	Protein Arrays
Separation	Electrophoresis: IEF PAGE	Electrophoresis: IEF PAGE	LC/LC of peptides	LC of peptides	Binding of proteins based on their chemical and physical characteristics	Antibody-based chips (binding to affinity reagent)
Quantification	Densitometry of stains	Densitometry of Cy3 and Cy5 labeled proteins normalized to Cy2	None	Through use of heavy and light tags	Comparison of MS peaks	Densitometry of binding
Identification	Mass spectrometry (PMF)	Mass spectrometry (PMF)	Mass spectrometry (MS/MS)	Mass spectrometry (MS/MS)	Difficult, requires serial of sample or coupling to second MS instrument	Binding to particul ar affinity reagent
Hydrophobic proteins	Dependent on detergents used	Dependent on detergents used	Theoretically better than electrophoresis but not systemicall examined	No better than 2-DE	Moderate	Unknown
Low expressed proteins	Marginal	Moderate (especially with scanning gels)	Moderate, often used with large sample amounts	Moderate	Marginal to moderate	Unknown
Requirement for protein identification	No	No	No	No	No	Yes
Potential for discovering novel proteins	Yes	Yes	Yes	Yes	Yes	No
Detection of specific protein isoforms	Yes	Yes	Yes	No	No	Yes
Relative assay time	Moderate	Moderate	Rapid	Rapid	Rapid	Rapid
Cost to acquire	Cheap (30,000–40,000$)	Expensive (180,000–200,000$)	Moderate (50,000–60,000$)	Moderate (40,000–50,000$)	Expensive (150,000–180,000$)	Cheap (10,000–20,000$)
Cost to used	Cheap	Expensive	Expensive	Expensive	Expensive	Cheap
Advantages	Well-established method. Powerful to detect protein modifications Low cost	Good quality of quantification of multiple samples. Total number of gels for an experiment reduced in regard to traditional 2-D E. Powerful to detect protein modifications.	Excellent approach if no quantification is desired. Significantly higher sensitivity than 2D techniques (much larger coverage of the proteome for biomarker discovery)	Designed for quantification Theoretically, higher coverage of proteome. Simultaneity for protein identification	Ability to bind a range of proteins to different molecular surfaces without the requirement for antibody production. Easiest MS instrumentation	High throughput
Limitations	Possible biased for the quantification according the stains used. Each stain has a specific dynamic range and a specific sensitivity	Requires expensive dedicated instrumentation and labeling reagents. Risk of nonlinear dynamic range of fluorescent dyes like observed recently in differential levels of mRNA (transcriptome)	No quantification Requires high level of MS skill Complicated data compilation	Requires high level of MS skills. Complicated data compilation. At each experiment only two treatments were compared	Some problems of reproducibility and repeatability. Difficult to identify proteins	Specificity of antigen/antibody binding. Queries on quantitative accuracy

Note: 2-DE: two-dimensional electrophoresis; 2-DIGE: two-dimensional difference in gel electrophoresis; MuDPIT: multidimensional protein identification technology; ICAT: isotope coded affinity tags; SELDI-TOF MS: spectrum-enhanced laser desorption ionization–time of flight mass spectrometry; IEF: isoelectric focusing; PAGE: polyacrylamide gel electrophoresis; LC/LC: tandem liquid chromatography; LC: liquid chromatography; PMF: peptide mass fingerprint; MS/MS: tandem mass spectrometry.

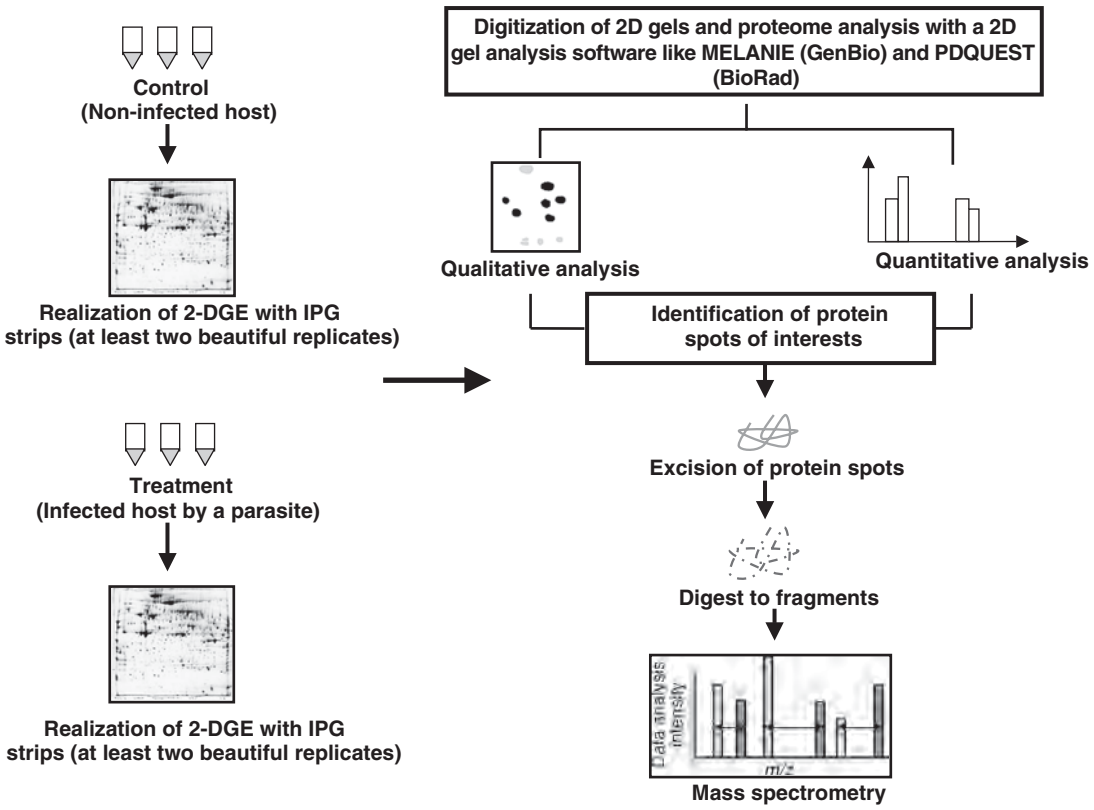

Fig. 21.4. Classical approach in "parasito-proteomics" project.

disease, tuberculosis, and human fungi using 2-DE gels has led to the discovery of several new and unexpected antigenic peptides, including housekeeping enzymes involved in energy metabolism, amino acid biosynthesis, or general cellular processes [67–69,96,99]. The cell proteome is clearly very dynamic and proteomic approaches facilitate the detection of changes in protein composition under different conditions.

Other studies have revealed the limitations of the current approach in parasito-proteomics by showing that in the host–parasite interaction, many immune mechanisms are involved (constitutive, induced, specific, or otherwise) [48,76,120]. By using two treatments, the injection of lipopolysaccharides (LPS) and a sterile injury, Vierstraete et al. [120] were able to disentangle proteome modifications induced by immunity from those induced by a physical stress. Levy et al. [76] studied the immune response of the fruitfly, *Drosophila* to bacterial (*Micrococcus luteus* and *Escherichia coli*), and fungal (*Beauveria bassiana*) infections. The data revealed that 70 of the 160 protein spots detected were differentially expressed at least fivefold after a bacterial or fungal challenge. In addition, the majority of these spots were specifically regulated by one pathogen, whereas only a few spots corresponded to proteins altered in all cases of infection.

In summary, the current approach in parasito-proteomics has many benefits in terms of understanding fundamental aspects of gene–protein functional interactions. Unfortunately, it is not applicable to different parasite species (and as such, does not encourage the growth of knowledge of general host proteome responses), nor does it necessarily help in the creation of a proteomic database with a holistic relevance to the understanding of host–parasite interactions.

21.4.1.3 Toward a new conceptual approach Some proteomics studies have shown common features in the innate response of plants, insects, and mammals [18,20,107,115]. The plant defense response is mediated by disease resistance genes (R genes), which are abundant throughout the genome and confer resistance to many microorganisms, nematodes, and/or insects [27]. *R* genes of several families of plants studied to date show homology with the *Drosophila* receptor *Toll* and the mammalian interleukin-1 receptor [107]. In addition, plants, invertebrates, and vertebrates produce a class of peptides called "defensins" which are pathogen-inducible [18,54]. Some peptides and/or proteins used by phytophagous or animal parasites to modify the genome expression of their host share many structural and functional homologies. Thus, for example, phytoparasitic root-knot nematodes of the genus *Meloidogyne* secrete substances into their plant hosts in order to make a giant cell used as a feeding site [1,28]. A similar system is observed for the zooparasite, *Trichinella spiralis* (Stichosomida: Trichinellidae) [65]. Furthermore, the injection of a peptide isolated from nematode secretions to either plant protoplasts or human cells enhances cell division [44]. The mechanism is not yet well known, but protein induction is

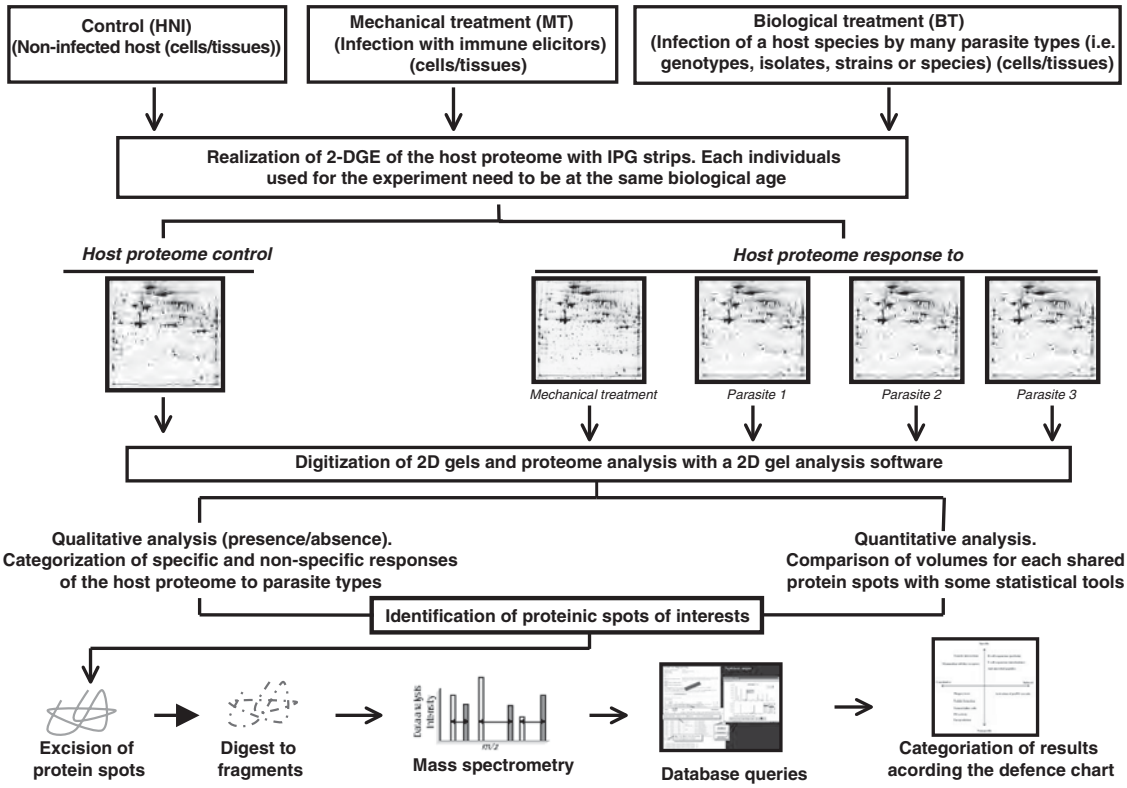

Fig. 21.5. A new methodological approach in "parasito-proteomics" taking into consideration the defense chart based on evolutionary ecology concepts of immune responses of host to parasite types with classical 2-DE techniques.

considered as a strong possibility. These days, many data are obtained by genomic and proteomics projects concerned with host–parasite interactions. Nevertheless, as mentioned above, generally little effort is made to elaborate such projects with respect to a holistic view of the goal to increase knowledge concerning immune responses of a host along with the biochemical cross-talk communication between host and pathogen/parasite.

In a recent article, we suggested a new, more holistic approach to parasito-proteomics researchers based on evolutionary concepts of immune responses of a host to an invading pathogen/parasite [14,15]. From an evolutionary ecological point of view, host immune responses to a particular parasite can be plotted on a chart according to the immune mechanisms used (constitutive vs. induced) and degree of specificity. The first axis of the defense chart refers to the immune mechanisms employed by the host with the two extreme cases: (i) a constitutive immune mechanism used by the host to rapidly impair the invasion by a parasite; and (ii) an induced immune mechanism which has the advantage of avoiding a costly defense system, yet meanwhile has the disadvantage that the parasite might escape host control [111]. The second axis of the defense chart refers to the degree of specificity of the host immune response.

Whatever the tactics used and the degree of specificity, the host genome ensures the adequate operation of the immune response via the proteome (genome operating system). For each immune tactic, many proteins are implicated. Consequently, any researcher in parasito-proteomics working with the immune defense chart will be able to categorize the host genome reaction for any given parasite at any given time. This type of approach should be as much hypothesis generating for parasito-proteomics as for evolutionary ecology itself.

In Figure 21.5, such a novel methodological parasito-proteomics approach is detailed, taking in consideration the defense chart based on evolutionary ecology concepts of immune responses [111]. To attain this goal, the approach recommends following – for example – the temporal expression of the hemolymph proteome of the host in relation to three types of treatment: control, injection of immune elicitors, and infection by a given number of parasite species (see Fig. 21.5). This approach thus permits both qualitative (presence/absence) and quantitative analysis, such as heuristic and principal component analysis (PCA) on common protein spots along with cluster analysis on their intensity (Eisen method). The goal is to categorize the protein spots (according to the defense chart) following the host genomic responses upon invasion by different parasitic genotypes, isolates, strains, or species.

Two-dimensional analysis software such as ImageMaster2 Dtm Platinum Version 5.0 (Amersham Biosciences, UK; GENEBIO, Switzerland) takes into consideration gel variation in order to make a ratio for each common protein spot based

on the sum of volume (integration of optic density over a spot's area for all groups). The statistical method used to analyze the common protein spots is superior to that used in the classical approach, that is, the method does not compare only the difference among treatments. Instead, the host–parasite interaction is seen in its entirety (i.e., globally) by analyzing each treatment (gel) as a variable. In this way, not only is the global impact of parasite activity on the expression of the host proteome (genome operating system) observed but also the impact of parasite types.

Qualitative analysis, by identifying the induced (detected on gel) and suppressed (absent or not detected on gel) protein spots, helps in determining the specific and nonspecific responses of the host proteome following parasite invasion. Quantitative analysis based on the relative abundance and volume of common protein spots (groups) not only leads to the identification of common spots differentially expressed between treatments but also by comparison of the gels (treatments) as related to the total proteome. Our methodological approach favors both the finding of new biochemical mechanisms implied in the host immune response as well as the categorization of the protein families.

Heuristic analysis makes it possible to classify gel (treatments) into two or more groups and to determine the characteristic proteinic spots of each group, that is, the proteins which might be differentially expressed [8]. The visual task of comparing gels can be rather difficult when dealing with a large number of such gels displaying thousands of spots. In particular, it may be hard to assess different sample populations as well as characterize their different protein expression profiles. PCA may help here, by providing a way to condense the information contained in these huge data sets into a smaller number of dimensions that explain most of the variance observed. PCA can thus be used to examine the interrelationships among a large number of variables (e.g., spot values for a series of gels), and to explain these relationships (e.g., gel populations) in terms of some common underlying factors like association with specific spot patterns [15,80,101]. Lastly, cluster analysis adapted from Eisen et al. [31] and based on the intensity of each common protein spot, gives access to similarly expressed spots and/or differences between treatments. The intensity of each protein spot is assigned by the "Vol%" value observed divided by the average of "Vol%" observed for each group of common proteinic spots and the intensity values are centered and reduced inside the same gel. Proteinic spots with similar profile pattern of expression are grouped in the same cluster (group). This clustering method allows discrimination to the kinetic expression between treatments (2-DE gels), and with a color code showing the expression of proteinic spot in relation to the others of a same group (a proteinic spot shared by all treatments), namely, red for overexpression, green for lower expression, and black for the absence of differential expression.

21.4.1.4 Concluding remarks The new conceptual and methodological approach presented here will, we hope, contribute to accelerating knowledge of specific immune responses to different parasite species. At the same time, such an approach should lead to the creation of a proteomic database, thereby allowing a holistic view on host–parasite interactions based on evolutionary concepts of immune responses of host to a parasite. This methodological approach offers a new route not only to drug and vaccine discovery but also to the study of host–parasite interactions as seen following the characterization of proteins implicated in the alteration of the host behavior observed in many taxa [14]. It may also provide a way of reconstructing the molecular phylogeny of proteins involved in the host immune response and used to determine their level of conservation over evolutionary time scales.

21.4.2 Bioinformatics

21.4.2.1 A new emerging scientific field to address biological questions The term bioinformatics, like the even more general term "computational biology," is sometimes used to refer to the application of computational methods to the analysis or storage of any type of biological or biomedical data. Here, we prefer to use a somewhat more restricted definition, namely, bioinformatics is the computational analysis of the sequence, structure, and expression of biological macromolecules. Bioinformatics aims to extract information from data regarding biological macromolecules and to use that information to address biological questions.

The computational strategies employed by "bioinformatics" are in large part comparative, for example, they may involve the comparison of homologous sequences or structures. Thus, bioinformatics is inherently based on evolutionary biology. The information bioinformatics can obtain from molecular data has arisen as a direct result of the evolutionary processes inherent in the genome of the organisms studied. These processes include mutation, which is the source of all new sequence variation in natural populations, including not only point mutations but also more complex mutational changes such as gene duplication, rearrangements, and deletions. Other important processes are genetic drift and natural selection, which can lead to the elimination or fixation of new variants.

The roots of bioinformatics extend back into the pregenomic era. One important root is population genetics, a topic theoretically explored in depth by Motoo Kimura, who, in the 1950s, first elucidated the dynamics of genetic drift and its interaction with natural selection. Kimura's work culminated in the synthesis which he called the "Neutral Theory of Molecular Evolution" [71]. This theory forms the current theoretical basis for our understanding of molecular evolution and thus constitutes the theoretical framework on which bioinformatics depends.

Although the development of bioinformatics would not have been possible without recent advances in the availability and affordability of powerful computers, it is worth recalling that bioinformatics began at a time which was not only "pre-genomic" but also to a large extent pre-computer!

Perhaps, the seminal paper in bioinformatics was Zuckerkandl and Pauling's [137] discussion of the potential for comparative study of protein sequences. Even though they did not use a computer, these authors showed that protein sequences may be aligned and that by studying the differences between aligned sequence positions, important biological information can be obtained. A second key development in the pre-genomic era was Margaret Dayhoff's realization in the 1970s of the need to gather the slowly but steadily increasing number of sequences in a database that could be – and as it turned out, would be – periodically updated. Though initially distributed in a printed rather than a digital version, this sequence database, which later became known as the Protein Information Resource (PIR), introduced a key component of bioinformatics as known today.

Bioinformatics has already had a major impact on our understanding of infectious diseases, and will doubtless continue to play a crucial role in the future. Here, we briefly discuss some of the important aspects of the biology of infectious agents where bioinformatics analysis is proving highly illuminating as we enter the post-genomic era. We discuss the implications of genome-wide bioinformatics approaches for the understanding of the following aspects of parasite biology: (i) polymorphism and population structure of pathogen species; (ii) the role of natural selection on parasite genes, particularly selection exerted by the host immune system; (iii) the role in recombination between genomes; and (iv) the analysis of gene and protein expression data in an evolutionary context.

21.4.2.2 Polymorphism and population structure

When a new genome is sequenced, it is routine to announce that the genome of such-and-such species has been sequenced. But, of course, there is no such thing as "the" human genome or "the" genome of any species for that matter. Rather, because of sequence polymorphism, each species is comprised of many different genomes. One of the dangers of the post-genomic era is that biologists will neglect the study of genetic polymorphisms and the important biological insights that they can provide. To molecular biologists, questions of population genetics may often seem rather arcane, but in the case of pathogens, these questions may have major practical importance.

A parasite species with a large effective population size and, consequently, substantial genetic variation may be much more difficult to eradicate than a parasite that is more genetically uniform. Vaccines based on parasite antigens may not be effective against all genotypes of the parasite if there are polymorphisms at antigen-encoding loci. In addition, any type of chemical prophylaxis or drug treatment imposes strong selection on the parasite. A genetically diverse parasite should be more capable of responding to such selection than a genetically uniform one [63].

An accurate assessment of genetic polymorphisms in parasite species requires an extensive amount of data. Indeed, in many cases, this question is one that can only be addressed in a post-genomic context, with complete genome sequences available for comparison. The example of a major human pathogen illustrates why this is so. In the 1990s, sequencing of individual genes from the bacterium, *Mycobacterium tuberculosis*, the etiologic agent of tuberculosis in humans, showed surprisingly little polymorphism, suggesting that the worldwide population of this microorganism might be nearly uniform genetically and derived from a single, very historically recent ancestor. Particularly striking was the very low level of synonymous polymorphisms observed. However, within a few years, the complete genomes of two isolates of the bacterium were available. When compared, a substantial degree of synonymous polymorphism was observed, although still far lower than seen in populations of several other pathogenic bacteria [62].

Simple considerations of probability can explain why earlier studies produced relatively low estimates of diversity in *M. tuberculosis*. The mean proportion of synonymous sequence differences observed between the two completely sequenced isolates, H37Rv and CDC1551, was about three differences per ten thousand sites [62]. If we assume that the per site probability of a synonymous difference between two *M. tuberculosis* genomes is equal to this proportion, then the probability that no synonymous differences will be seen in a gene with 150 synonymous sites is about 95%. The probability that no synonymous differences will be seen in ten such loci chosen at random is about 60%, and the probability that no synonymous differences will be observed at 20 such loci is about 37%. On the other hand, the probability that no synonymous differences will be seen at 100 such loci is less than 1%.

A similar example is provided by the most virulent human malaria parasite, *P. falciparum*. Rich et al. [103] found no synonymous differences at a small sample of loci from *P. falciparum* drawn from GenBank entries. However, a more extensive sampled a few years later showed ample evidence of synonymous polymorphisms [63]. Moreover, a study involving numerous single nucleotide polymorphisms (SNPs) on chromosome 2 of *P. falciparum* showed substantial polymorphisms and thus evidence for a large effective population size [88]. The latter study, in particular, demonstrates the power of a new technology – in this case, the genotyping of SNPs – as applied at a large scale to resolve previously controversial biological questions.

21.4.2.3 The role of natural selection

Since the seminal writings on evolution by Charles Darwin (1809–1882) and Alfred Russel Wallace (1823–1913) in the late 1850, biologists have invoked natural selection as a mechanistic explanation for the origin of adaptations. Natural selection is predicted to play a particularly important role in the evolution of parasites, as these organisms will be subject to strong selection-favoring mechanisms concerned with evading detection and elimination by host immune defense mechanisms. Before the availability of molecular sequence data, the evidence for the role of natural selection was largely indirect. Sequence data have now, however, made it possible to

observe the effects of natural selection at the most fundamental level [57].

Numerous methods have been proposed to obtain evidence of natural selection at the molecular level. Many widely used approaches involve comparison of the pattern of synonymous and nonsynonymous (amino acid-altering) nucleotide substitutions in gene coding regions [57]. In most genes, the number of synonymous nucleotide substitutions per synonymous site (dS) exceeds the number of nonsynonymous substitutions per nonsynonymous site (dN). This pattern is evidence of purifying selection acting to eliminate deleterious nonsynonymous mutations. By contrast, a pattern of dN exceeding dS is evidence that positive Darwinian selection is acting to favor amino acid changes.

Not surprisingly, some of the best-documented examples of positive selection at the molecular level involve gene regions that encode epitopes recognized by the immune system of the vertebrate host. For example, in vertebrates, the class I major histocompatibility complex (MHC) and cytotoxic T cells (CTL) interact to identify and kill cells infected by any intracellular parasite, including viruses, certain bacteria, and protists. This recognition gives rise to selective pressure favoring CTL escape mutants on the part of the parasite – mutations that change the epitope in such a way as to eliminating binding by the host MHC.

Some of the best evidence for such selection comes from experimental infections of rhesus monkeys (*Macaca mulatta*) with simian immunodeficiency virus (SIV) [32]. In these experiments, all monkeys were inoculated with the same virus, and the evolution of the virus over the course of infection was monitored. Because the monkeys were of known MHC type, it was possible to compare evolution of the same genomic region in a monkey in which that region encoded a CTL epitope with those in which it does not. Figure 21.6 shows mean dS and dN for comparisons between the inoculum and complete viral genomes taken at time of death from 35 SIV-infected monkeys [90]. Mean dS and dN were computed separately for nonepitope regions; for regions that encode known CTL epitopes but not those presented by the infected monkey's MHC ("nonrestricted epitopes"); and for regions encoding epitopes presented by the monkey's MHC ("restricted epitopes"). There was a significant difference in mean dN among regions, with the highest dN in restricted epitopes, but no such difference was seen in mean dS (Fig. 21.6). These results show that the genomic regions encoding peptides recognized by the host immune system are subject to positive selection favoring amino acid changes—a pattern not seen in other regions.

21.4.2.4 Recombination Recombination mechanisms may be important in the evolution of pathogens because they enable the exchange of genes that confer virulence or resistance to antibiotics or drugs. The exchange of plasmids bearing genes for antibiotic resistance has been a major factor in the recent evolution of bacterial pathogens. The availability of complete genome sequences for many bacterial

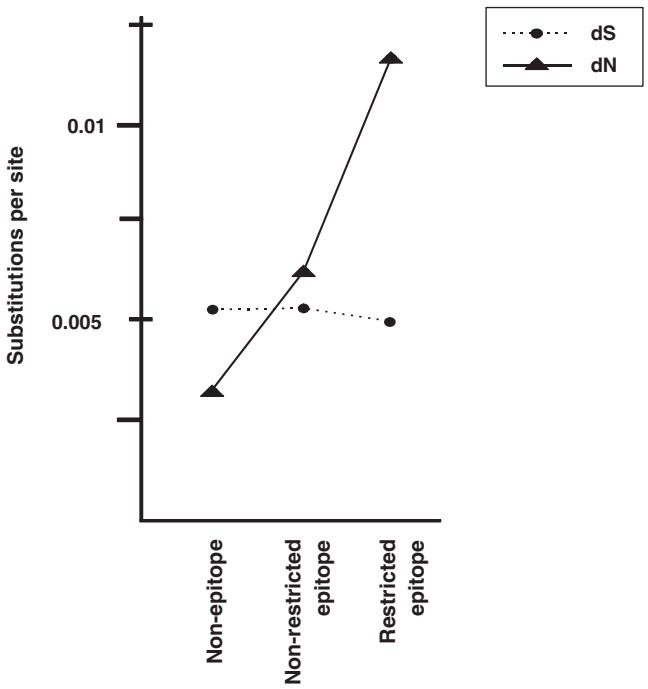

Fig. 21.6. Mean numbers of synonymous substitutions per synonymous site (dS) and of nonsynonymous substitutions per nonsynonymous site (dN) in difference regions of SIV complete genomes (data from [90]).

pathogens makes it possible to examine recombination at a genome-wide level. Most obviously, such comparisons can reveal genes present in one isolate but absent in another. For example, comparisons of complete genomes of different *Staphylococcus aureus* isolates have illustrated that, in addition to a conserved core of shared genes, different isolates have independently acquired different sets of large mobile elements carrying genes responsible for virulence or drug resistance [55]. The best studied such element is the staphylococcal cassette chromosome mec (SSCmec) element, which confers resistance against the β-lactam family of antibiotics [25,52].

Apart from the acquisition of new genes, recombination between two bacterial genomes can involve the exchange of sequence at orthologous loci. Thus, a typical bacterial genome is often a kind of a mosaic with regard to the evolutionary histories of its genes. Hughes and Friedman [59] examined such recombination by computing dS in pairwise comparisons among orthologous genes from five complete genomes of *S. aureus*. The parameter dS was used to provide a measure of evolutionary divergence – roughly proportional to the time since the most recent common ancestor had two alleles in homozygous condition at a particular locus, because synonymous sites are expected to be less subject to natural selection than nonsynonymous sites.

Certain genes were found to show very high dS between certain pairs of genomes, one or two orders of magnitude higher than the dS value between the same pair of genomes at a typical locus [59]. Such a high value of dS indicates that

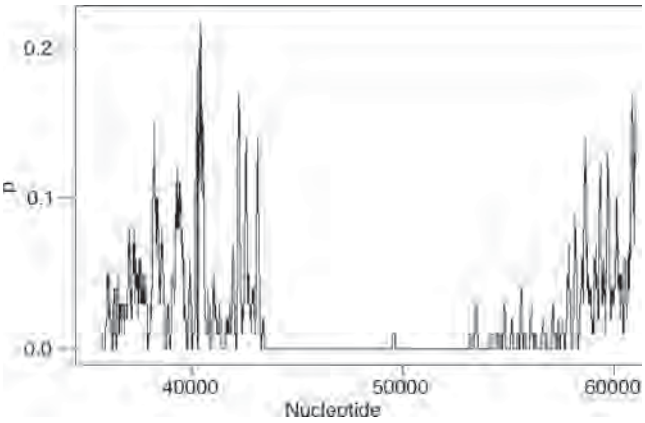

Fig. 21.7. Plot of the proportion of nucleotide difference (p) between two genomes of *Streptococcus agalactiae* in a sliding window of 100 nucleotides across the genomic region including the "pur" genes. Nucleotide position of the starting point of each window on the X-axis is numbered as in the 2603V/R genome (NC_004116) (positions 35756-61023) (from [54]).

the locus in question was subject to homologous recombination that introduced a divergent sequence into one or both of the genomes. Alignments of entire genomes or of extensive genomic segments, including many genes, can reveal large segments in which two genomes are highly divergent, whereas in other segments, they are virtually identical. Figure 21.7 shows an example from two genomes of *Streptococcus agalactiae* [58].

21.4.2.5 Analysis of gene and protein expression data

Genomics and proteomics are important tools by which to enhance understanding of biological processes, but both techniques generate large amounts of data that are often difficult to analyze [73,112]. One frequent problem in such studies is the limited amount of replication. This often makes it impossible to decide whether an observed difference represents a real biological effect or is simply due to random fluctuation or experimental error. An evolutionary perspective can assist in such analyses. One example of an evolutionary approach involves grouping genes in multigene families prior to analyzing expression data [60]. In multicellular organisms, a substantial proportion of genes are members of multigene families. Analysis of expression differences between and within families can provide a natural way of grouping genes that makes both statistical testing possible as well as providing insights into the evolutionary processes by which duplicate genes have differentiated functionally [60].

Another way that evolutionary analysis can shed light on gene expression data is to compare patterns of sequence evolution in order to observe expression differences. For example, it is possible to test how expression patterns are correlated with the rate of nonsynonymous evolution in the coding sequence. Hughes and Friedman [61] examined the relationship between gene expression across the life cycle and protein conservation in *P. falciparum* by comparing gene expression data for six life history stages with the number of nonsynonymous substitutions per site (dN) between 901 orthologous gene pairs of *P. falciparum* and *P. yoelii*. A high level of expression across the life history was associated with decreased dN and thus with protein conservation. By contrast, differential expression in the sporozoite and merozoite stages was associated with increased dN. This pattern probably reflects both positive selection acting on surface antigens of these infective stages [64] and a general trend toward reduced functional constraint on proteins with narrow expression [134].

21.4.3 Post-Genomic Era and Bioterrorism

Following the events of "nine eleven" and the subsequent postal anthrax attacks in the United States, the possibility of further bioterrorism attacks became globally all too real. As a direct consequence of this, the US government expanded its "biodefense" program, with studies ranging from basic research to applications in detection, prevention, and treatment of diseases caused by such microbiological agents, the net result of which has been great progress in understanding their genomics [34]. Efforts were focused on the three major categories of critical biological agents classified by the CDC (http://www.bt.cdc.gov/agent/agentlist-category.asp#catdef). In the post-genomic era, the benefit of having the full sequence of the genomes of these agents is obvious. There are now genome sequences for a few isolates of each species, which have made studies of comparative genomics a reality and which are leading to important discoveries – such as the diversity of closed related isolates and the identification of new putative virulence genes [39]. In this way, breakthrough transcriptomic and proteomic studies promise further exciting results and surprises over the next few years, which hopefully will have highly beneficial applications in terms of combating the scourge of global bioterrorism (see also the chapter by Morse, this volume).

21.5 CONCLUSION

From the dawn of human evolution to the influenza and HIV/AIDS pandemics of the twentieth and early twenty-first centuries, infectious diseases have continued to emerge and reemerge with great ferocity and by so doing, seriously affect populations as well as challenge our abilities to combat the agents responsible [33,84]. Over the past decade, strains of many common microbes have continued to develop resistance to the drugs that once were effective against them [33,84]. In the battle against infectious diseases, humankind has created new mega-technologies such as massive sequencing, proteomics, and bioinformatics, but without conceptual approaches based on the evolutionary concepts. Parasite genome sequences do not of themselves provide a full explanation of the biology of an organism and the dynamic genome interactions in a host–parasite system [2,132]. In this chapter, new ways based on evolutionary

concepts are suggested, more especially proteomics and bioinformatics, to enable further elucidation of the molecular complexities of host–parasite genome interactions. The new conceptual approach suggested for parasito-proteomics will help to increase the knowledge of immune response to different parasite species, in addition to the creation of a proteomic database. The latter will also enable a holistic view of host–parasite genome interactions to be taken based on evolutionary concepts of host immune response to an infectious agent. In addition, it will open the way to reconstructing the molecular phylogeny of proteins such as those involved in the host immune response and to determine their level of conservation during evolution.

ABBREVIATIONS

2-DE: Two-dimensional electrophoresis
2-DIGE: Two-dimensional in gel electrophoresis
AFLP: Amplified fragment length polymorphism
AIDS: Acquired immune deficiency syndrome
AP-PCR: Arbitrarily primed polymerase chain reaction
ASAP: Arbitrary signatures from amplification profiles
CDC: Centers for Disease Control and Prevention
CTL: Cytotoxic T cells
dN: Number of nonsynonymous substitutions per nonsynonymous site
dS: Number of synonymous nucleotide substitutions per synonymous site
FBAFLP: Fluorescence-based fragment length polymorphism
HIV: Human immunodeficiency virus
ICAT: Isotope-coded affinity tags
IEF: Isoelectric focusing
ISSR: Inter simple sequence repeats
LC: Liquid chromatography
LC/LC: Tandem liquid chromatography
LPS: Lipopolysaccharides
MALDI-MS: Matrix-assisted laser desorption/ionization–mass spectrometry
MHC: Major histocompatibility complex
MS: Mass spectrometry
MS/MS: Tandem mass spectrometry
MST: Multilocus sequences typing
MuDPIT: Multidimensional protein identification technology
PAGE: Polyacrylamide gel electrophoresis
PCA: Principal component analysis
PCR: Polymerase chain reaction
PCR-RFLP: Polymerase chain reaction–restriction fragment length polymorphism
PFGE: Pulsed-field gel electrophoresis
p*I*: Isoelectric point
PIR: Protein information resource
PMF: Peptide mass fingerprint
RAHM: Random amplified hybridization microsatellites
RAMP: Random amplified microsatellite polymorphism
REA: Restriction endonuclease analysis
RFLP: Restriction fragment length polymorphism
SCAR: Sequence-characterized amplified region
SELDI-TOF MS: Spectrum enhanced laser desorption ionization–time of flight mass spectrometry
SIV: Simian immunodeficiency virus
SNPs: Single nucleotide polymorphisms
S-SAP: Sequence-specific amplification polymorphism
SSCmec: Staphylococcal cassette chromosome mec
SSCP: Single-strand conformation polymorphism
SSR: Simple sequence repeats

GLOSSARY

AIDS: Acronym for acquired immune deficiency syndrome, the gravest of the sexually transmitted diseases, or STDs. It is caused by the human immunodeficiency virus (HIV), now known to be a retrovirus, an agent first identified in 1983. HIV is transmitted in body fluids, mainly blood and genital secretions.

Antigen: Substances that are foreign to the body and cause the production of antibodies.

Biochemical cross-talk: Molecular communications in host /parasite system during their interaction.

Bioinformatics: The computational biology is the use of mathematical and informational techniques, including statistics, to solve biological problems, usually by creating or using computer programs, mathematical models, or both. One of the main areas of bioinformatics is the data mining and analysis of the data gathered by the various genome projects. Other areas are sequence alignment, protein structure prediction, systems biology, protein–protein interactions, and virtual evolution.

Chemical prophylaxis: The administration of chemicals or drugs to members of a community to reduce the number of carriers of a disease and to prevent others contracting the disease.

Cytotoxic T cells: A subset of T lymphocytes that can kill body cells infected by viruses or transformed by cancer.

Defensin: A substance with natural antibiotic effects found in human blood cells. There are three types of defensins. Other animal species have similar substances.

Elicitors: Molecules produced by the host (or pathogen) that induce a response from the pathogen (or host).

Epitopes: An epitope is the specific site on an antigen to which an antibody binds.

Genetic drift: Random changes in the frequency of alleles from generation to generation; especially in small populations, can lead to the elimination of a particular allele by chance alone.

Genomics: It is the study of an organism's genome and the use of the genes. It deals with the systematic use of genome

information, associated with other data, to provide answers in biology, medicine, and industry.

Genome: The full complement of genes carried by a single (haploid) set of chromosomes. The term may be applied to the genetic information carried by an individual or to the range of genes found in a given species.

Major histocompatibility complex (MHC): Two classes of molecules on cell surfaces. MHC class I molecules exist on all cells and hold and present foreign antigens to CD8 cytotoxic T lymphocytes if the cell is infected by a virus or other microbe. MHC class II molecules are the billboards of the immune system. Peptides derived from foreign proteins are inserted into MHC's binding groove and displayed on the surface of antigen-presenting cells. These peptides are then recognized by T lymphocytes so that the immune system is alerted to the presence of foreign material (see Histocompatibility Testing).

Mass spectrometry: A technique for separating ions by their mass-to-charge (m/z) ratios.

Neutral theory of molecular evolution: The Japanese biologist M. Kimura (1924–1994), as a result of his work on population genetics and molecular evolution, developed a theory of neutral evolution which opposed the conventional neo-Darwinist theory of evolution by natural selection. He received many honors for his work, including Japan's highest cultural award, the Order of Culture.

Orthologous genes: Groups of genes or proteins from different organisms that have the same function are said to be orthologous. Homologous sequences are said to be orthologous when they are direct descendants of a sequence in the common ancestor (i.e., without having undergone a gene duplication event).

Paralogous: Two homologous sequences (e.g., sequences that share a common evolutionary ancestor) that diverged by gene duplication, as opposed to orthologs, which diverged by speciation. Parameters are user-selectable values, typically experimentally determined, that govern the boundaries of an algorithm or program. For instance, selection of the appropriate input parameters governs the success of a search algorithm. Some of the most common search parameters in bioinformatics tools include the stringency of an alignment search tool, and the weights (penalties) provided for mismatches and gaps.

Parasito-proteomics: The study of the reaction of the host and parasite genomes through the expression of the host and parasite proteomes (genome-operating systems) during their biochemical cross-talk.

Pathogenicity: The capability of a pathogen to cause disease.

Polymorphism: Variation in a region of DNA sequence among different individuals; the variation should be present in at least 1–2% of the population to be considered a polymorphism.

Population genetics: Population genetics is the area of *Genetics* that studies the distribution of genes (the units of genetic inheritance) and genotypes (the genetic complement at one or more loci), and the mechanisms determining genetic variability within a population.

Principal component analysis: It is a descriptive/exploratory technique designed to analyze two-way and multi-way tables containing some measure of correspondence between the rows and columns. The results provide information which is similar in nature to those produced by factor analysis techniques, and they allow one to explore the structure of categorical variables included in the table.

Proteome: The term proteome was first used in 1995 and has been applied to several different types of biological systems. A cellular proteome is the collection of proteins found in a particular cell type under a particular set of environmental conditions such as exposure to hormone stimulation. It can also be useful to consider an organism's complete proteome. The complete proteome for an organism can be conceptualized as the complete set of proteins from all of the various cellular proteomes. This is very roughly the protein equivalent of the genome. The term "proteome" has also been used to refer to the collection of proteins in certain subcellular biological systems. For example, all of the proteins in a virus can be called a viral proteome.

Proteomics: The large-scale study of proteins, particularly their structures and functions. This term was coined to make an analogy with genomics, and is often viewed as the "next step," but proteomics is much more complicated than genomics.

Rhesus: Group of antigens on the surface of red blood cells of humans which characterize the rhesus blood group system. Most individuals possess the main rhesus factor (Rh+), but those without this factor (Rh-) produce antibodies if they come into contact with it. The name comes from rhesus monkeys, in whose blood rhesus factors were first found.

Simian immunodeficiency virus: It is a virus of the genus Lentivirus, closely related to human immunodeficiency virus that causes inapparent infection (presence of infection in a host without the occurrence of recognizable symptoms or signs) in African green monkeys and a disease resembling acquired immunodeficiency syndrome in macaques.

Synonymous: When used in connection with coding sequences in nucleic acids, the term refers to different nucleotide sequences which code for the same amino acid. So, for example, the mRNA sequences GUU and GUC both code for valine. A mutation in the third position from U to C is a synonymous change as it results in the same protein. Changes in the third codon position are usually synonymous.

Transcriptome: Is the whole set of mRNA species in one or a population of cells.

Transcriptomics: Techniques to identify mRNA from actively transcribed genes.

Two-dimensional gel electrophoresis: Proteomics, the study of the proteome, has largely been practiced through the separation of proteins by two-dimensional gel electrophoresis. In the first dimension, proteins are separated by isoelectric focusing, resolved on the basis of charge. In the second dimension, they are separated by molecular weight using SDS-PAGE. To visualize the proteins, the gel is dyed with Coomassie Blue, silver, or other reagents. Spots on the gel are proteins that have migrated to specific locations.

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CHAPTER 22

Mathematical Modeling of Infectious Diseases Dynamics

M. Choisy,[1,2] J.-F. Guégan,[2] and P. Rohani[1,3]

[1]*Institute of Ecology, University of Georgia, Athens, USA*

[2]*Génétique et Evolution des Maladies Infectieuses UMR CNRS-IRD, Montpellier, France*

[3]*Center for Tropical and Emerging Global Diseases, University of Georgia, Athens, USA*

"As a matter of fact all epidemiology, concerned as it is with variation of disease from time to time or from place to place, must be considered mathematically (. . .), if it is to be considered scientifically at all. (. . .) And the mathematical method of treatment is really nothing but the application of careful reasoning to the problems at hand."

—Sir Ronald Ross MD, 1911

22.1 INTRODUCTION

The concealed and apparently unpredictable nature of infectious diseases has been a source of fear and superstition since the first ages of human civilization (see Chapters 31 and 40). The worldwide panic following the emergence of SARS and avian flu in Southeast Asia are recent examples that our feeling of dread increases with our ignorance of the disease [48]. One of the primary aims of epidemic modeling is helping to understand the spread of diseases in host populations, both in time and space. Indeed, the processes of systematically clarifying inherent model assumptions, interpreting its variables, and estimating parameters are invaluable in uncovering precisely the mechanisms giving rise to the observed patterns. The very first epidemiological model was formulated by Daniel Bernoulli in 1760 [11] with the aim of evaluating the impact of variolation on human life expectancy. However, there was a hiatus in epidemiological modeling until the beginning of the twentieth century[1] with the pioneering work of Hamer [32] and Ross [54] on measles and malaria, respectively. The past century has witnessed the rapid emergence and development of a substantial theory of epidemics. In 1927, Kermack and McKendrick [41] derived the celebrated threshold theorem, which is one of the key results in epidemiology. It predicts – depending on the transmission potential of the infection – the critical fraction of susceptibles in the population that must be exceeded if an epidemic is to occur. This was followed by the classic work of Bartlett [9], who examined models and data to expose the factors that determine disease persistence in large populations. Arguably, the first landmark book on mathematical modeling of epidemiological systems was published by Bailey [8] which led in part to the recognition of the importance of modeling in public health decision making [7]. Given the diversity of infectious diseases studied since the middle of the 1950s, an impressive variety of epidemiological models have been developed. A comprehensive review of them would be both beyond the scope of the present chapter and of limited interest. Instead, here we introduce the reader to the most important notions of epidemic modeling based on the presentation of the classic models.

After presenting general notions of mathematical modeling (Section 22.2) and the nature of epidemiological data available to the modeler (Section 22.3), we detail the very basic *SIR* epidemiological model (Section 22.5). We explain

[1]During the nineteenth century research activity on infectious diseases was dominated by the clinical studies at the Pasteur school.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

the assumptions made about the biological processes and their consequences from an epidemiological perspective. We then review more complex models that allow the study of endemic diseases (Section 22.6) and recurrent epidemics (Section 22.7). Section 22.8 then focuses on the analysis of epidemiological data and the estimation of model parameters. The chapter ends with some examples of practical uses of models for the development of public health policies (Section 22.9). Technical aspects are treated in boxes.

22.2 THE PHILOSOPHY OF MATHEMATICAL MODELING

Epidemiology is essentially a population biology discipline concerned with public health. As such, epidemiology is thus heavily influenced by mathematical theory. The reason is that most phenomena observed at a population level are often complex and difficult to deduce from the characteristics of an isolated individual. For example, the prevalence of a disease in a population is only indirectly connected to the course of disease in an individual. In this context, the use of mathematical models aims to unearth processes from a large-scale perspective.

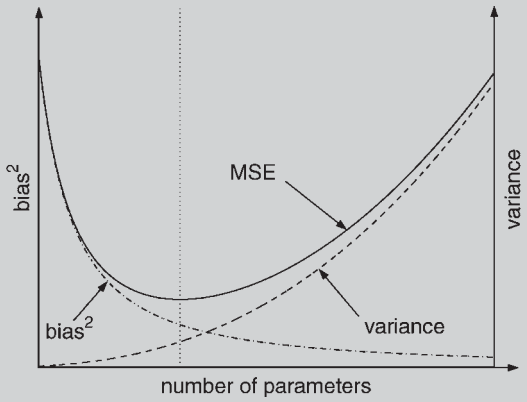
22.2.1 Model Complexity

A model is a caricature of reality as represented by empirical data. Models help us to understand reality because they simplify it. Consequently, all models are by definition "wrong." There are, however, models which more closely capture essential features of reality than others – we usually refer to these as better fitting data. There is a temptation to assume that only models that are incredibly detailed (and hence "realistic") can be useful – this is often not the case, however. A model should only be as complex as needed, depending on the questions of interest. This philosophy is referred to as Occam's razor or the principle of parsimony and can be summarized as *the simplest explanation is the best*.

We now proceed to introduce some of the modeler's vocabulary. A state variable is a changing quantity that characterizes the state of the system. For example, the number of infectives and susceptibles in the population are state variables of an epidemiological system. The modeler is interested in the behavior of the state variables. A parameter is a user-defined quantity that influences the value of the state variables. For example, the average duration an individual stays infectious is a parameter of an epidemiological system. The fit of a model to a data set is basically influenced by two aspects [35]. The first is related to the complexity of the model as given by the number of variables and parameters. Complicated models will usually give better fits to data than simpler models. However, simpler models are more transparent and often provide insight that is more valuable and influential in guiding thought. The choice of the optimal level of complexity obeys a trade-off between bias and variance [14] (see Box 22.1). The second aspect is related to the exact relationship between the parameters. For example, should the transmission process be linear or nonlinear? Again, it is important to realize that the nature of such a relation does not need to be totally correct for the model to be useful. Modelers speak of structural stability, which refers to whether small changes in the model assumptions result in substantial changes in prediction.

BOX 22.1 – HOW COMPLEX SHOULD A MODEL BE?

With the current power of desktop microcomputers it is tempting to build very complex models in order to fit the data the most. However, fitting the most complex model is not necessary always the best solution. Indeed, the more complex a model, the more difficult the interpretation of its outputs. Also, if a model is too complex, the modeler may not have sufficient information in the data to distinguish between the possible parameter values of the model. As said in the main text, the best-sized model depends on the purpose of the model. Given this objective, there exist quantitative methods for determining the optimal size of a model. These approaches are based on a trade-off between prediction error due to approximation (i.e., bias) which decreases as model complexity increases, and prediction error due to estimation (i.e., variance) which increases as model complexity increases as shown in the figure below [14]. The consequence is that for any model and amount of data, the total prediction error (proportional to the mean squared error) will decrease and then increase as model complexity increases, thus evidencing an optimal level of model complexity.



The mean squared error (MSE) is equal to MSE = variance + bias^2. As the number of parameters increases the bias decreases and the variances increase, defining an optimum number of parameters corresponding to the minimum of the MSE, as materialized by the vertical dotted line in the above figure.

22.2.2 Model Formulation and Hypothesis Testing

A mathematical model is a set of equations, which are the mathematical translation of hypotheses (or assumptions). When interpreting model predictions, it is thus important to bear in mind the underlying assumptions. By definition, an assumption is an unverified proposition, tentatively accepted to explain certain facts or to provide a basis for further investigation. For example, one can construct a model 1, assuming that the probability a susceptible gets infected is proportional to the number of infectives and a model 2 assuming that this probability is independent of the number of infectives. In such an instance of competing hypotheses, the data can act as an arbitrator by telling which model is more consistent with the data [59]. In modern statistics the fit of a model to a data set is measured by its likelihood (see Box 22.2). Comparison of models is thus based on the comparison of their likelihoods. As the likelihood of a model naturally increases as the

BOX 22.2 – LIKELIHOOD FUNCTIONS

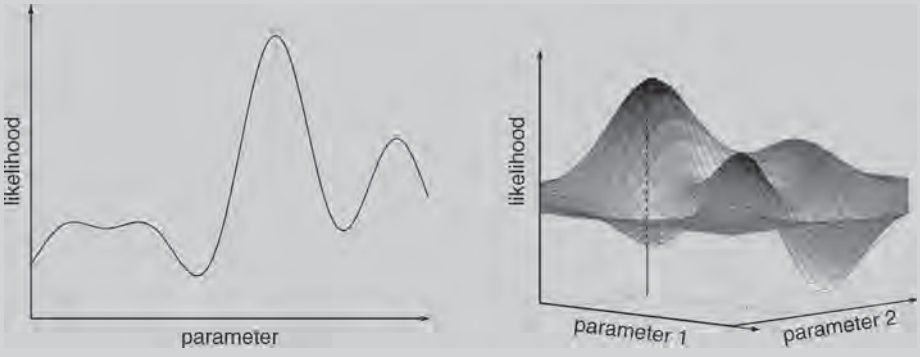
The likelihood of a model is a measure of the probability that the model is the appropriate description of the reality, given the data: L(model | data) = Pr(model | data). One powerful point of the likelihood function is that the term "model" includes not only the mean trend but also the variance, that is the distribution of the errors around the mean trend. Whereas the classical least square method implicitly assumes a normal distribution of errors, the likelihood methods allow considering any error distribution. For example, suppose that **d** is a vector of data and **m** a vector of model predictions with a mean trend depending on one parameter x. Assuming now that the errors are normally distributed with a variance σ^2 then the likelihood of one prediction of the model reads

$$L(\mathbf{m}_i(x),\sigma^2 \mid \mathbf{d}_i) = \frac{1}{\sqrt{2\pi\sigma^2}} \exp \frac{(\mathbf{d}_i - \mathbf{m}_i)^2}{2\sigma^2}$$

If the vector of data is a time series – as often the case for epidemiological data – then the data points are not independent. However, if the noise has a large magnitude – as often the case for epidemiological data too – the approximation of independency between the data points becomes acceptable. In that case the likelihood function of the model reads

$$L(\mathbf{m}(x), \sigma^2 \mid \mathbf{d}) = \prod_i L(\mathbf{m}_i(x), \sigma^2 \mid \mathbf{d}_i) = \prod_i \frac{1}{\sqrt{2\pi\sigma^2}} \exp \frac{(\mathbf{d}_i - \mathrm{m}_i)^2}{2\sigma^2}$$

We thus end with a function which depends on two parameters x and σ^2. This likelihood function can be used for two different purposes. First, this function can be used to estimate parameters x and σ^2, good estimations of them being values that maximize the likelihood function as shown in the figures below with one and two parameters.



If the search of the maximum of the likelihood is straightforward when the function depends on one parameter, it becomes more complicated when the number of parameters increases. Microcomputers now allow the use of efficient numerical algorithm to find the maximum of such multiple dimensional surfaces. Among the most popular are the Newton and the Nelder-Mead algorithms [51]. Second, the expression of a likelihood function allows the comparison of different competing models, using either the likelihood ratio test or the Akaike information criterion (see main text).

number of its parameters increases (see above Section 22.2.1), it is necessary that the likelihood comparisons correct for the complexities of the models. There exist two major procedures for model likelihood comparison [37].

In a classic null hypothesis approach, the likelihood ratio test (LRT) is the most commonly used procedure. Two models are said nested when one is a particular case of the other. Twice the difference between log-likelihoods of two nested models follows a χ^2 distribution with degree of freedom equal to the difference between the numbers of parameters of the two models. A more complex is thus retained if its likelihood is significantly higher than the one of a simpler model, as judged from the χ^2 statistic.

We can alternatively use model selection criteria to rank any (nested or not) competitive models. These criteria are basically constructed as a likelihood value corrected for the complexity of the model. The most used of these criteria is the Akaike information criterion (AIC) [2] defined as AIC = 2(p-LL), where p is the number of parameters and LL is the logarithm of the likelihood.

22.2.3 Stochastic Versus Deterministic Models

Deterministic models are those in which there is no element of chance or uncertainty. As such, they can be thought to account for the mean trend of a process only. Stochastic models, on the other hand, account not only for the mean trend but also for the variance structure around it. In an epidemiological context, there are two main kinds of stochasticity: demographic and environmental. Demographic stochasticity reflects the fact that while all individuals may be subject to the same possible events with the exact same probabilities, chance events may result in differences in the fates of individuals. When a phenomenon is the sum of a large number of small individual effects (as disease propagation in large population), the weak law of large numbers diminishes the effects of demographic stochasticity and a deterministic model becomes appropriate. In contrast, when the population is small, random events cannot be neglected and a stochastic model is necessary. Environmental stochasticity refers to the situation where there is variation in the probability associated with an event. Consequently, some parameters of stochastic models may be uncertain and characterized by a probability distribution instead of a constant value. For fixed starting values, a deterministic model will always produce the same result whereas a stochastic model will produce many different outputs, depending on the actual values the random variables take.

22.3 THE NATURE OF EPIDEMIOLOGICAL DATA

Epidemiology is fundamentally a data-driven discipline, and a key element in this research field is being able to link mathematical models to data. Epidemiological data are generally based on the disease notifications reported by medical doctors, veterinarians, or agronomy engineers. Epidemiologists usually consider incidences defined as the number of new cases per unit of time and prevalences referring to the number of diseased people, ideally at one instant, and in practice over a short period of time. Incidences thus reflect the dynamics of the disease whereas prevalence is more related to the static properties of the disease. Epidemiological data may further be stratified by age, sex, social status, geographical location, and so on. In Section 22.6.2 we will see that stratification by age is of particular interest as age reflects time [7]. Moreover the survey can be carried out longitudinally (i.e., through time) or horizontally (i.e., at one instant or over a short period of time). In the first case, where the data are in the form of a time series, it is important to realize that the data of the series are not independent. Indeed, the number of new cases reported in a given week is likely to be close to cases reported during the previous week. Consequently, the statistical analysis of time series requires the use of specific tools presented in Section 22.8.2. Epidemiological data sets are often accompanied by demographic data such as the population size and the per capita birth rate in different localities and at different dates. This is of primary interest as the endemic state of an infectious disease is often dependent on host social and demographic factors.

Such data sets currently exist for a variety of diseases, in different locations and over several decades. Some of these data bases are available from the Internet, as the one used to draw Figures 22.6, 22.10, and 22.12. Other data sets can easily be requested from governmental health services. The quality of the data set is often related to its accuracy in terms of disease diagnose, spatial location, and notification frequency (weekly, monthly, or yearly).

22.4 CHILDHOOD MICRO-PARASITIC INFECTIONS

There exist a variety of epidemiological models and an exhaustive review of them cannot be performed in one single chapter. As a result, we will focus our attention on some of the most frequently used models in order to highlight the general approaches and the main results. We will thus be specifically interested here in childhood micro-parasitic infections. The distinction between microparasites and their counterpart macroparasites is not clear-cut and actually reflects more the way they are modeled than biological realities [7]. However, microparasites tend to refer to small-size parasites (viruses, bacteria, or protozoan) with fast and direct reproduction within the host. Childhood microparasitic diseases usually transmit by direct contact through droplets and the infectivity is generally high. The host usually recovers from the infection and acquires immunity for some time (often for life). The disease generation length (i.e., the duration between the infection and the clearance by the host immune system) is generally short relative to the host life expectancy. Because of the fast and direct reproduction within the host, it makes sense to model the dynamics of microparasitic diseases according to the host clinical status with compartmental models. We call childhood diseases those diseases which confer a lifelong

immunity. As the infectiousness of microparasitic diseases is usually high, the lifelong immunity makes the mean age at infection generally low, hence the name. Common childhood microparasitic infectious diseases include measles, rubella, chickenpox, mumps, whooping cough, and so on.

22.5 A SIMPLE EPIDEMIC MODEL

The idea behind compartmental models is to divide the host population into a set of distinct classes, according to its epidemiological status. One simple such model is the *SIR* formalism which classifies individuals as *S*usceptible to the disease (S), currently *I*nfectious (I), and *R*ecovered (R). The total size of the host population is then $N = S + I + R$. For childhood diseases there is no vertical transmission and thus individuals are born in the susceptible class (after any period of maternally derived immunity is passed). Upon contact with an infectious individual, susceptibles may get infected and move into the infectious class. Once the immune system clears the infectious agents, infecteds become immune and move to the recovered class (Figure 22.1).

22.5.1 Transmission Process

The transmission process is at the heart of any epidemiological model. To describe it, epidemiologists usually consider the force of infection λ defined as the per capita rate of acquisition of the infection. More precisely, $\lambda(t)\Delta t$ is the probability that a given susceptible individual will acquire the infection in the small interval of time Δt [34].

For airborne disease, the tradition has long been to consider the force of infection proportional to the number of infectious individual: $\lambda = \alpha I$. There is thus an analogy with the concentration of two chemical agents to which the law of mass action applies. However, humans obviously do not behave in exactly the same way as molecules in solutions as the daily contact patterns of people are often similar in large and small communities [34, 46].

Consider instead that the average number of contacts of a person per unit time is the constant β combining a multitude of epidemiological, environmental, and social factors that affect transmission rates [7]. Among these contacts, the number of contact with infectives is thus $\beta I/N$. Assuming that contacts are sufficient for transmission, the number of new cases per unit time is then $S\beta I/N$. Thus, in this case $\lambda = \beta I/N$, instead of $\lambda = \alpha I$. Fits to real data have proved that

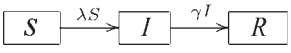


Fig. 22.1. A simple SIR epidemic model. The host population is divided into three compartments, according to their epidemiological status: susceptibles (S ind.), infectives (I ind.), and recovered (R ind.). Individuals move to the susceptible class to the infective class, to the recovered class according to the arrows. λ is the force of infection, that is, the probability that a susceptible individual gets infected, and γ is the recovery rate.

the frequency-dependant transmission process $\lambda = \beta I/N$ is more appropriate for human airborne diseases than the density-dependant one $\lambda = \alpha I$ [7]. The parameter α has no clear epidemiological interpretation but can be related to β as $\alpha = \beta/N$. McCallum et al. [46] explored other forms of the transmission process, including nonlinear ones, and studied their influence on the epidemiological conclusions.

22.5.2 Between-Compartment Flux of Individuals

A common assumption is that the movements out of one compartment into the next one are governed by constant rates [7]. For each time unit a constant number of individuals leave one compartment to the next, regardless to the time they spent in their compartment. The choice of this assumption is essentially motivated by an ease of mathematical tractability in a deterministic setup with ordinary differential equations. However, the assumption of a flux of individual at a constant rate r corresponds to exponentially distributed waiting times in the compartments. The parameter of the negative exponential distribution is r and thus the mean of the distribution $1/r$ (see Box 22.3). Analysis of real data reveals instead that each individual tends to spend a constant duration in each compartment [39, 43]. Models accounting for such realistic distributions of waiting times would imply the use of more sophisticated mathematics such as integro- or delay-differential equations. For didactic reasons we will here restrict our attention to the simplest and most used models based on simple ordinary differential equations and refer the reader interested in more realistic ones to [43] and [39]. Keeling & Grenfell [39] and Wearing et al. [60] showed that the assumptions on the waiting times can strongly influence the model outputs.

22.5.3 Basic Reproduction Number and Threshold Effects

One of the most fundamental quantities used by epidemiologists is certainly the basic reproduction number R_0. For microparasites it is defined as the expected number of secondary cases following the introduction of one infectious individual into a fully susceptible population [7]. We understand from here that R_0 has a threshold value in the sense that a disease must have $R_0 > 1$ to invade a host population, otherwise it disappears right after its introduction. The replacement number R is the average number of secondary infections produced by a typical infective during its entire period of infectiousness. At the introduction of one infective into a fully susceptible population $R = R_0$ and then R decreases. At endemic equilibrium we will have, by definition, $R = 1$ (see Sections 22.6.1 and 22.9.1.1).

22.5.4 Deterministic Setup and Dynamics Analysis

For large populations, deterministic models with continuous variations of population sizes provide a good description of the disease behavior. Epidemic models are used to describe

BOX 22.3 – MODELING THE INFECTIOUS PERIOD

In the classic *SIR* model it is usually assumed that the individuals leave the infectious class at a constant rate. Even if this assumption seems the most intuitive, it is not always the most realistic in terms of the duration individuals stay infective. In this box we detail the consequences of the constant recovery rate assumption on the distribution of the infective periods, and propose an alternative which yields more realistic distributions [39,43,60].

Our random variable is the time of recovery since the infection. For discrete random variables (*e.g.,* number of individuals) it is easy to define a probability distribution $\Pr\{Z = k\} = f_k$ (as in Section 22.5.5) and then to define a cumulative distribution function $F(z) = \Pr\{Z \leq z\}$. For continuous variables, like here, the time of recovery since infection, it is impossible to define a probability of each time as there is an infinity of such times. The approach is then to first define a cumulative distribution and then express a probability density function from this cumulative distribution. The idea is to consider the probability associated with a short interval Δz of the random variable z.

$$\begin{aligned}\Pr\{z \leq Z \leq z + \Delta z\} &= F(z + \Delta z) - F(z) \\ &= F'(z)\Delta z + o(\Delta z)\end{aligned}$$

The derivative $F'(z)$ of the cumulative distribution $F(z)$ is by definition the probability density function.

Let us now apply this method to the time of recovery since the infection. As done in Section 22.6.3, we can express the probability of an infective to recover in the time interval Δt as

$$\Pr\{\text{recovery in } (t, t + \Delta t] \,|\, \text{no recovery in } (0, t]\} = \gamma\Delta t + o(\Delta t)$$

where t is the time since infection and γ is a fixed constant. The cumulative distribution is defined as $F(t) = \Pr\{\text{no recovery in } (0,t]\}$. For an infective not to recover in the interval $(0, t + \Delta t]$, he must first not recover in the interval $(0,t]$ and then not recover in the next Δt. Assuming that these events are independent gives

$$\begin{aligned}F(t + \Delta t) &= F(t)[1 - \gamma\Delta t + o(\Delta t)] \\ \Leftrightarrow \frac{F(t + \Delta t) - F(t)}{\Delta t} &= -\gamma F(t) + \frac{o(\Delta t)}{\Delta t}\end{aligned}$$

Taking the limit as $\Delta t \to 0$ gives

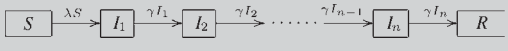
$$\frac{dF}{dt} = -\gamma F(t)$$

which, after integration and setting $F(0) = 1$ (i.e., no recovery before the infection), yields

$$F(t) = e^{-\gamma t}$$

Thus, infectious periods are exponentially distributed with a mean infectious duration equal to $1/\gamma$ (see dashed curve on the figure below). Inspecting real data, it seems that the infectious period does not follow an exponential distribution but rather seems to be of constant duration. To account for such more realistic distributions, we need to relax the assumption that the probability of recovery does not depend on the time since infection. There are several ways to do that, including integro-differential and partial differential formulations, but the simplest one is certainly the method of stages.

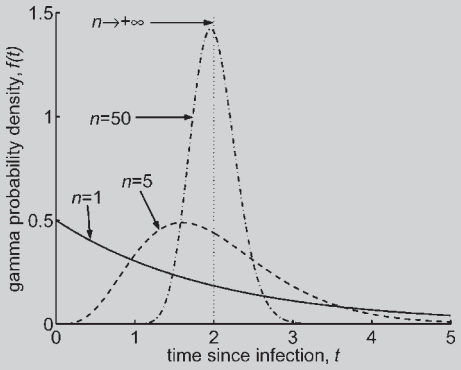
The basic idea of the method of stages is to replace the infective compartment by a series of n successive infective compartments, each with an exponential distribution of the same parameter:



The total duration of the infectious period is thus the sum of n identical and independent exponential distributions, which leads to a gamma distribution of the infectious durations:

$$f(t) = \frac{(\gamma n)^n}{\Gamma(n)} t^{n-1} e^{-\gamma n t}$$

where $\Gamma(n)$ is the gamma function. The variance of such a distribution is $1/(n\gamma^2)$. Notice that when $n = 1$ we find back the above-presented exponential distribution, when n gets large, the gamma distribution tends toward a normal one and when $n \to \infty$ we have the delta (fixed duration with no variance) distribution (see figure below).



The above figure shows gamma distribution for $\gamma = 0.5$ and various values of the number n of classes. When $n = 1$ we have the exponential distribution and when n increases the distribution tends toward a normal one. Ultimately, when $n \to \infty$ the gamma distribution converge toward the delta distribution with a variance equal to zero. Note that for all values of n the mean is equal to $1/\gamma = 2$.

rapid outbreaks that occur in very short periods of time, during which the host population can be assumed to be in a constant state [18, 19]. A mathematical description of the fluxes of individuals of Figure 22.1 is given by the following set of differential equations:

$$\frac{dS}{dt} = \beta S \frac{I}{N} \qquad S(0) = S_0 \geq 0 \qquad (22.1)$$

$$\frac{dI}{dt} = \beta S \frac{I}{N} - \gamma I \qquad I(0) = I_0 \geq 0 \qquad (22.2)$$

$$\frac{dR}{dt} = -\gamma I \qquad R(0) = R_0 \geq 0 \qquad (22.3)$$

where γ is the recovery rate. Since the duration of the epidemic is short, this model has no host vital rate. In consequence, the total host population size $N = S + I + R$ is constant and only two of the above equations are necessary to totally account for the disease behavior. Dividing the first two Equations (22.1) and (22.2) by the constant host population size N yields

$$\frac{ds}{dt} = -\beta i s \qquad s(0) = s_0 \geq 0 \qquad (22.4)$$

$$\frac{di}{dt} = \beta i s - \gamma i \qquad i(0) = i_0 \geq 0 \qquad (22.5)$$

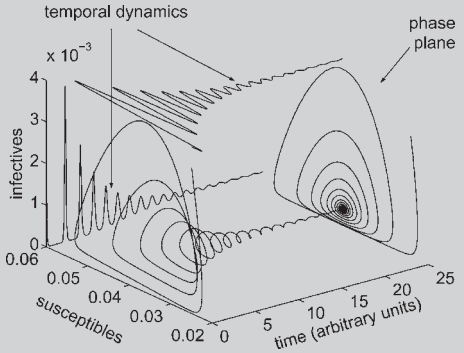
where $s(t) = S(t)/N$ and $i(t) = I(t)/N$. The basic reproduction number then reads $R_0 = s_0 \beta/\gamma$. Thus we can express the threshold on R_0 as follows. When $s_0 < \gamma/\beta$, on average each infective produces less than one infective and thus the number of infectives diminishes to reach 0 as time passes on. When $s_0 > \gamma/\beta$, the number of infectives first increases to then decrease toward 0, producing this characteristic epidemic peak. This threshold effect is illustrated on the phase plane (see Box 22.4) of Figure 22.2. We can see on this figure that when $s_0 < \gamma/\beta$ the proportion of infectives decreases toward 0, and when $s_0 > \gamma/\beta$ the proportion of infectives first increases to then decrease toward zero. In any case the proportion of infectives ends at zero whereas the ultimate value s_∞ of the proportion of susceptibles depends on the initial proportions s_0 and i_0 of susceptibles and

BOX 22.4 – GRAPHICAL TOOLS TO STUDY DYNAMICAL SYSTEMS

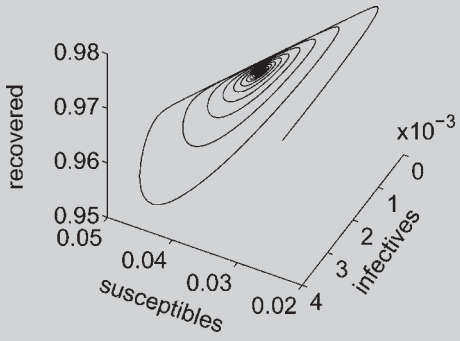
In this box we present two graphical tools facilitating the study of dynamical systems. The first one is the phase plane. Consider for example the endemo-epidemic *SIR* model of the main text

$$\begin{aligned} ds/dt &= \mu - \beta is - \mu s & s(0) &= s_0 \geq 0 \\ di/dt &= \beta is - \gamma i - \mu i & i(0) &= i_0 \geq 0 \end{aligned}$$

We can solve this system and draw the temporal dynamics of each of the state variable, $s(t)$, $i(t)$, and $r(t) = 1 - s(t) - i(t)$. A phase plane plots the behavior of one state variable as a function of another state variable. Temporal dynamics and phase plane are thus two different ways of visualizing the same reality as exemplified on the figure below.



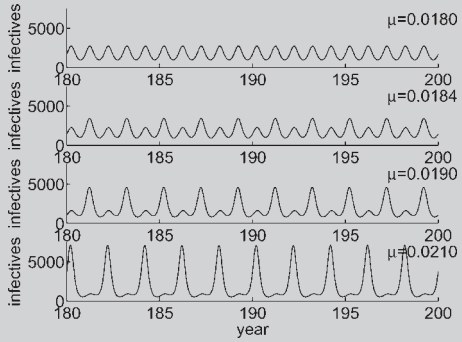
The phase plane of the above figure is the same as the one of Figure 22.7. There is no time dimension on phase planes but the trajectory of the dynamics is
usually indicated by arrows (see Figures 22.2, 22.5, and 22.7). A phase plane can also be drawn for three state variables like on the figure below.



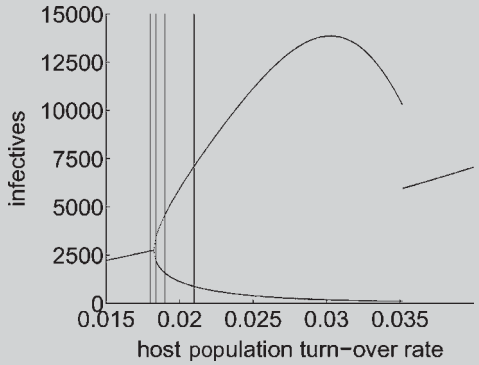
The second tool is relative to the complexity of a dynamics. Consider the same *SIR* model but now with a varying contact rate to sustain the oscillations (see Section 22.7.1):

$$\beta(t) = \beta_0(1 + \beta_1 \cos(2\pi t)) \qquad 0 \leq \beta_1 \leq 1$$

Running this model with different values of the host population turnover rate μ yields qualitatively different disease dynamics: on the figure below the dynamics changes from annual to bi-annual when μ increases from 0.0180 to 0.0210.



These qualitative changes on the dynamics are called bifurcations and the parameter we explore the influence (here μ) is called the control parameter. Bifurcation diagrams allow to visualize the effect of a control parameter on the complexity of the dynamics. For each value of the control parameter the simulated dynamics is sampled at regular time intervals. Imagine, for example, that we sample the dynamics every year. Then, an annual dynamics will give one point on the bifurcation diagram (each year the dynamics recovers the same value), whereas a biannual dynamics will give two points (one for the odd years and the other for the even years).



The above figure shows the bifurcation diagram of the disease dynamics with μ as the control parameter. The four vertical lines materialize the μ values corresponding to the above four time series. This diagram predicts that the disease dynamics is biannual for μ between 0.0183 and 0.0351 and annual for μ between 0.0150 and 0.0183 and between 0.0351 and 0.0400. At $\mu = 0.0183$ the switch from annual to biannual is progressive whereas at $\mu = 0.0351$ the switch from biannual to annual is sharp. Between 0.0183 and 0.0351 the disease oscillations reach their maximum at $\mu = 0.0303$.

infectives, respectively, as expressed by the following implicit equation [19]:

$$i_0 + s_0 - s_\infty + \log(s_\infty / s_0) / \sigma = 0 \tag{22.6}$$

We can see from Figure 22.2 that the higher the initial proportion of susceptibles s_0, the lower the proportion of individuals who do not get diseased during the epidemic. This is known as overshot phenomenon [19].

22.5.5 Stochastic Dynamics and Probability of an Epidemic in a Small Population

The deterministic *SIR* model presented above highlights a threshold value on the basic reproduction number with an epidemic when $R_0 > 1$ and no epidemic when $R_0 < 1$.

However, observations on real data reveal that $R_0 > 1$ does not guarantee an epidemic in the population [9]. The cause of this discrepancy between model prediction and observed data is that the deterministic *SIR* model is a good approximation of the epidemic dynamics only when dealing with large populations (see above Section 22.2.3), which is clearly not the case when we are interested in the initial epidemic growth following the introduction of one infective into a fully susceptible population. As the initial number of infectives during the initial epidemic growth is by definition very small, demographic stochasticity may play an important role in the start of an epidemic. The theory of branching processes is a useful framework to derive the probability that an epidemic starts [19].

Consider that the number of people infected by one infective follows a given probability distribution $\{q_k\}_k^\infty = 0$. Thus,

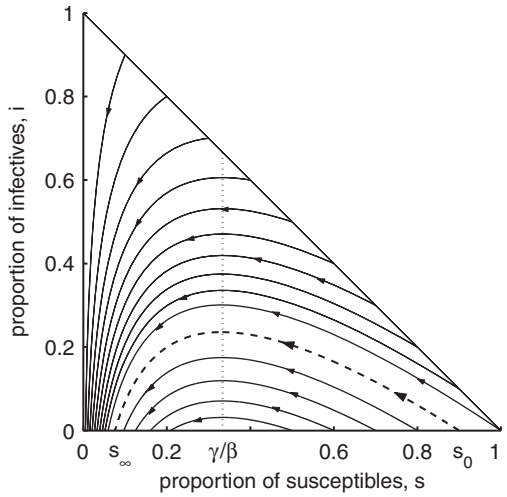


Fig. 22.2. Phase plane of the *SIR* epidemic model of Equations (22.4) and (22.5). The arrowed lines show the trajectories of the dynamics. The dashed arrowed line shows one particular trajectory with its initial and final proportions of suscpetible s_0 and s_∞, respectively. The vertical dotted line is the threshold γ/β on the value of the proportion of susceptibles (see main text). There is an epidemic only when s_0 is above this threshold.

any infective infects k individuals with the probability qk and $\sum_{k=0}^{\infty} q_k = 1$. The basic reproduction ratio R_0 can then be expressed simply as the expected number of individuals infected by one infective: $R_0 = \sum_{k=1}^{\infty} kq_k$. Then, we need to introduce the reader to one fundamental tool of branching processes: the generation function defined as

$$g(z) = \sum_{k=0}^{\infty} qkz^k \qquad 0 \le z \le 1 \qquad (22.7)$$

Among the interesting properties of the generating function are $g(0) = 0, g(1) = 1$, and $g'(1) = R_0$ [19]. Let z_n be the probability that the disease disappears from the population after n generations of transmission events. It can be shown that $z_n = g(z_n - 1)$ [33]. As the function g is increasing, the sequence z_n is increasing and tends toward a limit z_∞. By definition z_∞ is the probability that the disease introduced by one individual into a fully susceptible population will go extinct. Thus z_∞ is the solution of the equation $z = g(z)$. It can be shown that $z_\infty = 1$ for $R_0 \le 1$ and $0 < z_\infty < 1$ for $R_0 > 1$ [19]. Depending on the exact form of the infectious process, the solution z_∞ of the equation $z = g(z)$ can not always be expressed explicitly. For example, assuming that the number of infections during a constant time interval is according to a Poisson process, we end up with the following implicit expression of z_∞ 19]:

$$z = z\exp(R_0(z-1)) \qquad (22.8)$$

which can be easily solved graphically (see Figure 22.3).

22.6 A SIMPLE ENDEMIC MODEL

22.6.1 Deterministic Dynamics

Epidemic models presented in the above section are used to describe rapid outbreaks that occur in very short period of time, during which the host population can be assumed to be in a constant state. Such models thus do not need to account for the host population dynamics as governed by births and deaths. On longer period of times individuals will die and births will feed the population with new susceptibles, possibly allowing the disease to persist in the population at a low and constant prevalence. We then say that the disease is in an endemic state in the population [7]. If we are to study the endemic state of a disease, we need to construct a model that accounts for the birth and death rate of the host population. In the case of a nonfatal disease like most childhood ones in developing countries, a good approximation is to consider that the host population size $N = S + I + R$ is constant. The dynamics of the disease can then be described by the following differential

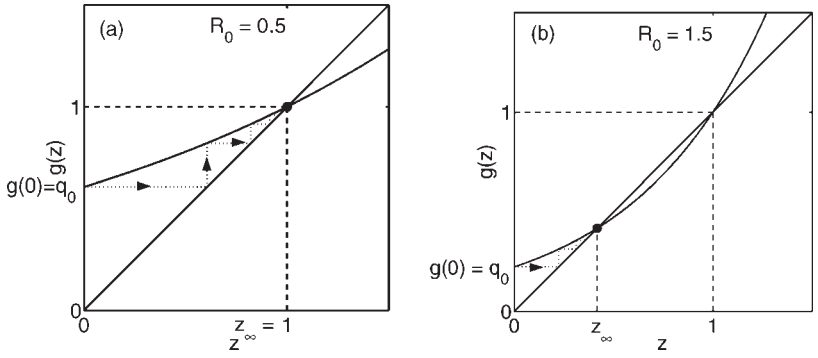


Fig. 22.3. Graphical resolution of the implicit Equation (22.8). Solutions of Equation (22.8) are the intersections between the first bissectrice and the curve which are respectively the l.h.s. and the r.h.s. of Equation (22.8), in the domain of definition [0,1]. $z_\infty = 1$ when $R_0 < 1$ (a) and $0 < z_\infty < 1$ when $R_0 > 1$ (b), (see main text).

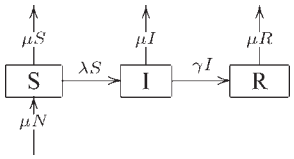


Fig. 22.4. A simple *SIR* endemic model. Same as Figure 22.1 except that now deaths remove individual from each compartment at a constant rate μ. Also, births feed the susceptible compartment with new individuals at the same rate μ. As the birth and death rates are equal, the total size N of the whole population remains constant, see main text.

equations which correspond to the flow diagram of Figure 22.4.

$$\frac{ds}{dt} = \mu - \beta is - \mu s \qquad s(0) = s_0 \geq 0 \qquad (22.9)$$

$$\frac{di}{dt} = \beta is - \gamma i - \mu i \qquad i(0) = i_0 \geq 0 \qquad (22.10)$$

where μ is the host population turnover rate, that is, the birth rate equal to the death rate. Again, for the simplicity of the mathematical analysis, assume that this rate has a constant value. As explained above and in Box 22.3, the consequence of this assumption is that the age distribution follows a negative exponential distribution. The mean of this distribution (i.e., the host life expectancy L) is equal to $L = 1/\mu$. This is a rather good approximation for the developing countries where the harshness of the environment imposes a similar death pressure on all the age classes [7]. However, in western countries, medical care allows most of the people to reach the natural age limit, yielding this characteristics square shape age distribution. Nevertheless, the exact form of the age pyramid does not have substantial influence on the dynamics of the disease [7].

The basic reproduction ratio now reads $R_0 = \beta/(\gamma + \mu)$. By definition, at equilibrium the system is in a constant state. Thus the differentials of Equations (22.9) and (22.10) should be equated to 0: $ds/dt = di/dt = 0$, which yields the following system of equations:

$$\mu - \beta is - \mu s = 0 \qquad (22.11)$$

$$\beta is - \gamma i - \mu i = 0 \qquad (22.12)$$

Solving this system produces two equilibrium points: (i) the disease-free scenario $(s_1^*, i_1^*, r_1^*) = (1, 0, 0)$ and (ii) the endemic case $(s_2^*, i_2^*, r_2^*) = (1/R_0, \mu(R_0 - 1)/\beta, 1 - s_2^* - i_2^*)$. The stability of these two equilibria depends solely on the value of the basic reproduction number, and not on the initial values of the proportions of susceptibles and infectives as in the above epidemic model. If R_0 is less than unity, then the disease-free equilibrium is stable [19] and the phase plane of Figure 22.5 shows that the proportion of susceptibles increases toward 1 whereas the proportion of infectives decreases toward 0. When $R_0 > 1$ means that the endemic equilibrium is stable [19] and Figure 22.5 shows that the proportions of susceptibles and infectives produce damped oscillations that converge toward their endemic values s^* and i^*. Linear stability analysis (see Box 22.5) reveals the natural period T and the damping time D of these damped oscillations to be approximated by

$$\hat{T} = 2\pi\sqrt{AG} \qquad (22.13)$$

and

$$\hat{D} = 2A \qquad (22.14)$$

respectively, where A represents the mean age at infection, $A \simeq 1/\mu(R_0 - 1)$ (see below Section 22.6.2), and G gives the ecological generation length of the infection, that is, the sum of the latent and infectious periods, $G = 1/(\mu + \gamma)$ [7, 53].

22.6.2 Statics and the Average Age at Infection

Once the endemic equilibrium is reached we may be interested in the statics of the disease such as the mean age at infection. This is of importance as, first, numerous diseases are

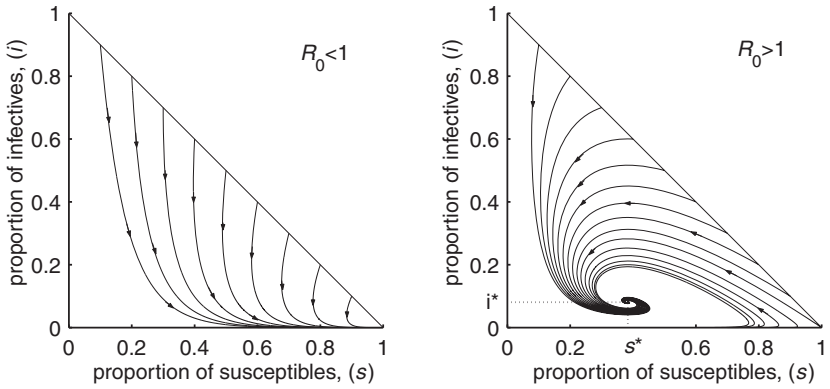


Fig. 22.5. Phase plane of the *SIR* endemic model of Equations (22.9) and (22.10). The arrowed lines show the trajectories of the dynamics. When the basic reproduction number $R_0 < 1$ the dynamics converges toward the stable disease-free equilibrium (left). When $R_0 > 1$ the disease dynamics converges toward endemic equilibrium (i^*, s^*) (right).

BOX 22.5 – LINEAR STABILITY ANALYSIS BASED ON EIGENVALUES

Linearization approximation is a standard phase plane technique used to analyze system dynamics [42]. For an *SIR* system with a constant host population size we have the following system of two independent nonlinear differential equations:

$$\frac{ds}{dt} = \mu - \beta is - \mu s \qquad s(0) = s_0 \geq 0$$

$$\frac{di}{dt} = \beta is - \gamma i - \mu i \qquad i(0) = i_0 \geq 0$$

As found in the main text, the endemic equilibrium of this system is $(s^*, i^*) = (1/R_0, \mu(R_0-1)/\beta)$. Close to the endemic equilibrium, the above system can then be rewritten into the following form:

$$s(t) = s^* + \xi(t)$$
$$i(t) = i^* + \zeta(t)$$

where $\xi(t)$ and $\zeta(t)$ are the deviations from the equilibrium. In order to study the stability of the equilibrium, we then need to focus on the dynamics of the deviations $\xi(t)$ and $\zeta(t)$ [42]. Combining the above two systems, developing, and keeping only the terms which are linear in ξ and ζ, we get

$$\frac{d\xi}{dt} = -(\beta i^* + \mu)\xi - \beta s^* \zeta + \mathrm{NL}(\xi, \zeta)$$
$$\frac{d\zeta}{dt} = \beta i^* \zeta + \mathrm{NL}(\xi, \zeta)$$

where $\mathrm{NL}(\xi,\zeta)$ contains all the nonlinear terms in ξ and ζ. Replacing s^* and i^* by their value gives

$$\frac{d\xi}{dt} = -[\mu(R_0 - 1) + \mu]\xi - \frac{\beta}{R_0}\zeta + \mathrm{NL}(\xi, \zeta)$$
$$\frac{d\zeta}{dt} = \mu(R_0 - 1)\xi + \mathrm{NL}(\xi, \zeta)$$

Written in matrix form, the above system becomes

$$\begin{bmatrix} d\xi/dt \\ d\zeta/dt \end{bmatrix} = \underbrace{\begin{bmatrix} -\mu(R_0-1) - \mu & -\beta/R_0 \\ \mu(R_0-1) & 0 \end{bmatrix}}_{\mathbf{J}} \cdot \begin{bmatrix} d\xi \\ d\zeta \end{bmatrix} + \mathrm{NL}(\xi, \zeta)$$

The Jacobian matrix **J** is called the community matrix in ecology and its eigenvalues are indicative of the dynamics of the system [42]. The eigenvalues of the community matrix are solutions of the characteristic equation

$$\Lambda^2 - \mathrm{Tr}(\mathbf{J})\Lambda + \det(\mathbf{J}) = 0$$

where Tr and det refer to the trace and the determinant of the matrix, respectively. Replacing the trace and determinant by their values gives

$$\Lambda^2 - \mu R_0 \Lambda + \mu(\mu + \gamma)(R_0 - 1) = 0$$

With the approximation pertaining to the fact that $\gamma \gg \mu$, we end with

$$\Lambda \simeq \frac{1}{2A} \pm j\frac{1}{\sqrt{AG}}$$

where A represents the mean age at infection, $A \simeq 1/\mu\ R_0$, and G gives the ecological generation length of the infection, that is, the sum of the latent and infectious periods, $G = 1/(\mu+\gamma) \simeq 1/\gamma$ [7,53]. The system oscillates with a period equal to 2π times the inverse of the imaginary part of the eigenvalue, $\hat{T} = 2\pi\sqrt{AG}$, and a damping time equal to the inverse of the real part of the eigenvalue, $\hat{D} = 2A$ [42].

in endemic state in human populations and, second, the study of static properties of a disease allows the estimation of key epidemiologic parameter without requiring the long series of longitudinal notifications, often difficult to obtain in practice. The idea behind studies on the statics of diseases is that the age of the individuals reflects, in some way, time [7]. What we simply need here is horizontal data stratified by age.

Considering the age as a continuous variable, the mean age at infection is simply expressed as [7]

$$A \equiv \int_0^\infty a \frac{\lambda s(a)}{\int_0^\infty \lambda s(a) da} da \qquad (22.15)$$

which is the integral sum of the age values a, weighted by the proportion of infectives of age a. Calculating this integral for a constant host population turnover rate μ yields the intuitive relationship $A = 1/(\lambda + \mu)$. This means that the higher the force of infection (i.e., the probability that a susceptible gets infected), the lower the mean age at infection. Moreover, recall from above (Section 22.6.1) that $i^* = \mu(R_0 - 1)/\beta$. Thus, $\lambda = \beta i^* = \mu(R_0 - 1)$. Rearranging this equation we get $\lambda + \mu = \mu R_0$. An expression of the mean age at infection then becomes $A = 1/(\mu R_0)$. This last expression allows estimating the basic reproduction number R_0 in a rather simple way as

$$R_0 = \frac{L}{A} \qquad (22.16)$$

where $L = 1/\mu$ is the host life expectancy (see Section 22.6.1).

22.6.3 Stochastic Dynamics and Disease Persistence

The above study of the deterministic dynamics of diseases has revealed a threshold on the value of the basic reproduction number. The disease immediately disappears after

its introduction as soon as $R_0 < 1$ and persists at an endemic level in the host population when $R_0 \geq 1$. However, by inspection of real data, it appears that the condition $R_0 \geq 1$ does not guarantee the disease persistence [9]. As already mentioned about the epidemic model (see Section 22.6.3), such persistence is dependent on the magnitude of the stochastic fluctuations around the endemic equilibrium.

In a metapopulation context, the probability of disease extinction in one subpopulation depends on both the size of the subpopulation and the fluxes of infectives from neighbor subpopulations. Bartlett [9] has thus evidenced that there is a community size above which the disease can be maintained in population by itself and below which the disease cannot persist in the population without regular fluxes of infectives from neighbor populations. The determination of this critical community size is performed empirically by plotting the mean annual duration of periods with no cases against the size of the subpopulation. By definition, we have a period of disease fade-out if the duration of the disease extinction is longer than the disease generation length [9]. Figure 22.6 shows an example for measles in 59 cities of England and Wales in the pre-vaccine era (1944–1966). For this disease the generation length is around 3 weeks and the critical community size is estimated here at about 115,000 individuals. The critical community size is thus a quantity very easily calculated from disease notifications and which gives a good idea of the population size required for disease persistence. Intuitively we expect that the more contagious a disease, the lower the critical community size. This explains why highly contagious diseases cannot persist in small isolated communities such as island populations or primitive Amazonian tribes.

22.7 ENDEMO-EPIDEMIC MODELS

So far we have seen simple models that allow the study of one isolated epidemic (Section 22.5) or of diseases in an endemic state (Section 22.6). However, it appears that numerous diseases are characterized by an endemic background with regular epidemics as visible on subplots of Figure 22.6 or on Figure 22.10. We say that these diseases are in an endemo-epidemic state in the population. In this section we propose some complications of the basic endemic model that allow producing recurrent outbreaks as observed on many longitudinal surveys.

In Section 22.6.1 we have shown that the endemic model exhibits damped oscillations which converge toward an endemic equilibrium. Linear stability analysis further revealed the natural period and the damping time of these oscillations to be approximated by $T = 2\pi\sqrt{AG}$ and $D = 2A$, respectively, where A and G are the mean age at infection and the disease generation length respectively (see Equations (22.13) and (22.14)). Importantly, for most epidemiologically reasonable parameter values, the damping time is typically much longer than the natural period: $2A/T \gg 1$. This renders the endemic equilibrium weakly stable, with relatively small per-

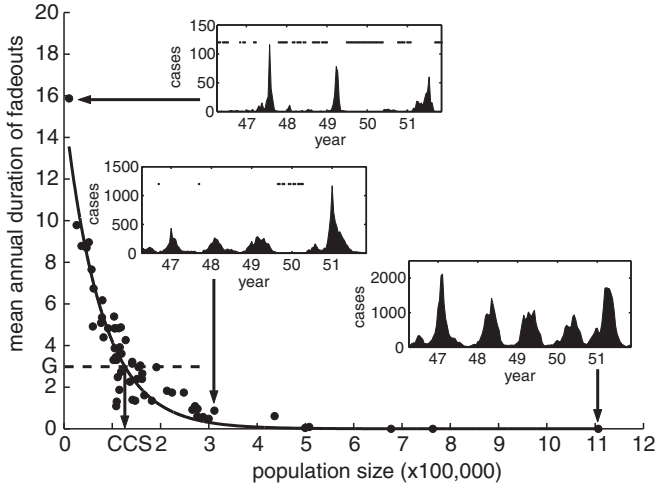


Fig. 22.6. Mean annual duration of fade-outs (i.e., local extinction) of measles against population size for 59 cities in England and Wales in the pre-vaccine era (1944–1966). Subplots show portions of time series illustrating the three levels of persistence identified by Bartlett [9]. Type I dynamics (bottom subplot: Birmingham, population of 1.1 millon ind.) are regular, endemic, with no fade-out. Type II dynamics (middle subplot: Nottingham, population of 300,000 ind.) are regular but with some fade-outs (represented by black dots) in the troughs. Type III dynamics (top subplot: Teignmouth, population of 11,000 ind.) are irregular with long fade-out between the epidemics. The curve is the nonlinear regression ($y \simeq 16 \times \exp[-10^{-5}\, x]$) and its intersection with the disease generation length (G, represented by the horizontal dotted line) gives the critical community size (CCS) of the disease of around 115,000 ind. Data downloaded from http://www.zoo.cam.ac.uk/zoostaff/grenfell/measles.htm [27].

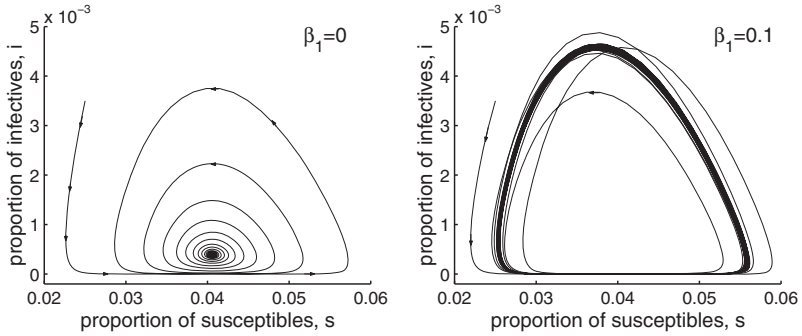


Fig. 22.7. Phase plane of the *SIR* endemo-epidemic model with a contact rate varying according to Equation (22.17) and $R_0 > 1$. When $\beta_1 = 0$, the dynamics converges toward an endemic equilibrium point (left). When $\beta_1 = 0.1$, the equilibrium point is destabilized and the dynamics produces sustained oscillations (right).

turbations (intrinsic or extrinsic) exciting and sustaining the inherent oscillation behavior [31]. Alternative mechanisms for this phenomenon have been proposed in the literature and all are based on the inclusion of some heterogeneity in the endemic model. Heterogeneity can be added temporally on the coefficient of transmission, spatially in the context of metapopulations, or by cohorts for age-structured models. Lastly, heterogeneity can be added statistically for full stochastic versions of the endemic model.

22.7.1 Varying Contact Rate

Temporal heterogeneity in the transmission rate was first proposed by Soper [56] who was attempting to explain the high amplitude outbreaks of measles in Glasgow. He demonstrated that transmission rates were high in October and declined through the academic year, with a trough in the summer months. This temporal variability in transmission rates, he argued, may be due to the considerably higher transmission rates when children are in school. Soper's conclusions were supported by later analyses of measles, chickenpox, and mumps in some US cities, as well as measles in England & Wales [26,44]. There has been a variety of mathematical forms proposed for taking into account seasonality in the coefficient of transmission [20, 22, 24, 40]. Certainly, the most realistic take the form of a binary function, with two different values of the coefficient of transmission – one for the school terms and one for the holidays. This necessitates the knowledge of the school holidays calendar which is not always possible, particularly for historical data. A simpler form of the coefficient of variation would simply take the form of a sinusoidal wave:

$$\beta(t) = \beta_0(1 + \beta_1 \cos(2\pi t)) \qquad 0 \le \beta_1 \le 1 \qquad (22.17)$$

where the strength of seasonality β_1 measures the amplitude of the oscillations around the baseline coefficient of transmission β_0. Although less realistic, this form of the coefficient of transmission produces results which are qualitatively very close to the ones obtained with a coefficient of transmission in plateau [22]. Figure 22.7 shows that even small strengths of seasonality are able to produce sustained oscillations.

22.7.2 Age-Structured Models

When the time is considered as a continuous variable, the most general form of the force of infection is actually the following [7]:

$$\lambda(a,t) = \int_0^\infty \beta(a_s, a_i, t)\, i\,(a_i, t)\, da_i \qquad (22.18)$$

where $\beta(a_s, a_i, t)$ is the coefficient of transmission between a susceptible of age a_s and an infective of age a_i at time t. In Sections 22.5 and 22.6 we averaged this relation over both time and ages. In the above Section 22.7.1 we averaged over ages only and we defined the coefficient of transmission as a function of time (see Equation (22.17)):

$$\bar{\lambda}(t) = \bar{\beta}(t)\bar{i}\,(t) \qquad (22.19)$$

where bars refer to age average. In the present section we average the relation 22.18 over time only. We thus have to define a coefficient of transmission as a function of age. Contrary to time, it does not really make biological sense to consider age as a continuous variable as human populations are usually aggregated by cohorts defined as the primary school children, the intermediate and high school teenagers, the college young adults, and the adults [7]. When averaging over time and considering the age variable as a discrete variable, Equation (22.18) becomes:

$$\hat{\lambda}_i = \sum_{j=1}^{n} \hat{\beta}_{i,j}\, \hat{i}_j \qquad (22.20)$$

where hats refer to time average and n is the number of distinct cohorts. We thus need to define a matrix of transmission $[\hat{\beta}_{i,j}]$.

TABLE 22.1. Transition Events, and Corresponding Rates, for a Simple Stochastic SIR Model

	Type of transition event	Rate	Event
1	$S \to S+1,\ I \to I,\ S \to R$	$r_1 = \mu N$	Birth
2	$S \to S-1,\ I \to I,\ S \to R$	$r_2 = \mu S$	Death
3	$S \to S,\ I \to I-1,\ S \to R$	$r_3 = \mu I$	Death
4	$S \to S,\ I \to I,\ \to S-1$	$r_4 = \mu R$	Death
5	$S \to S-1,\ I \to I+1,\ R \to R$	$r_5 = \beta IS/N$	Infection
6	$S \to S,\ I \to I-1,\ R \to R+1$	$r_6 = \gamma I$	Recovery
7	$S \to S,\ I \to I+1,\ R \to R$	$r_7 = \delta$	Immigration of infectives

22.7.3 Spatially Structured Models

The organization of human populations in distinct cities interconnected by fluxes of individuals makes the theory of metapopulations an appropriate framework to study the spatial dynamics of infectious diseases [28, 29]. In this context the definition of a spatially structured model is pretty close to an age-structured one. We thus need to define a matrix of transmission. Two common assumptions are that this matrix is symmetric and the values $\beta_{i,j}$ are related to the geographic distance between the cities i and j (Figure 22.8). However, given the speed of communication networks at a regional scale, a simple and widely used approximation of this metapopulation model is the island model in which all the subpopulations are linked the ones to the others by the same coupling coefficient ε:

$$\lambda_i = \beta \times \left((1-\varepsilon) i_j + \varepsilon \sum_{k \neq j} i_k \right) \qquad (22.21)$$

The $(1 - \varepsilon)$ term ensures that the basic reproduction ration R_0 stays constant. Other spatial models are not based on the theory of metapopulations and instead consider the spatial dimension as a continuous variable. Those models are based on the reaction–diffusion equations that, for simplicity, we will not treat in the present chapter.

22.7.4 Stochastic Endemic Models

Sections 22.5 and 22.6 have primarily focused on deterministic models, that is, models in which nothing is random. These models produce pretty good predictions as long as the population is large enough for the stochasticity to have little influence. However, in Sections 22.5.5 and 22.6.3 we highlighted that in small populations, deterministic model predictions become unreliable. To study disease dynamics in small populations, one thus often need a stochastic instead of a deterministic model [6]. In this section we present an easy way to construct a stochastic version of the *SIR* endemic model and we will show that stochasticity introduces enough heterogeneity in the model to produce sustained oscillations.

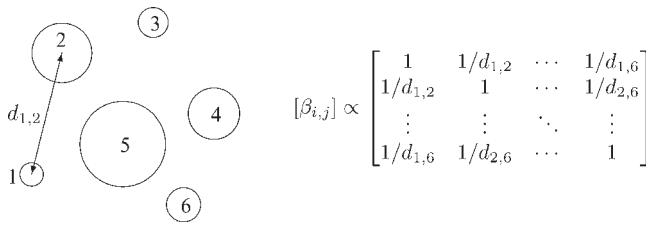


Fig. 22.8. In a metapopulation context the matrix of contact can be modeled as inversely proportional to the distance between the communities represented by circles.

A stochastic version of the endemic *SIR* model passes through the definition of a Markov process, that is, a process in which the future is independent of the past, given the present. The state space of this process is defined by the number of individuals in each of the three classes susceptibles (S), infectious (I), recovered (R). Changes in the state space are characterized by transition events which are listed in Table 22.1. Each transition event occurs with a probabilistic rate derived from the rates of the deterministic model. For example, the probabilistic rate corresponding to an event of birth is defined as follows:

$$\mathrm{P}\{1\,\text{birth in } (t, t+\Delta t] \mid S(t) = n\} = \mu n \Delta t + \mathrm{o}(\Delta t) \qquad (22.22)$$

$$\text{with } \lim_{\Delta t \to 0} \frac{\mathrm{o}(\Delta t)}{t} = 0.$$

For numerical simulation, the basic procedure consists in, first, searching the time of the next event (whatever its nature) and, second, determine the nature of this event. As all events are independent, the probabilistic rate that an event occurs, whatever its nature, is simply equal to the sum of the probabilistic rates of all the possible events $r = \sum_i r_i$. As future events are independent on past events, the time to the next event follows a negative exponential distribution of parameter r (see Box 22.3). Thus, the time to the next event can simply be determined by a random realization of a negative exponential probability distribution of parameter r. Then, the nature of this event is simply determined by a random realization of a multinomial probability distribution of parameters r_1/r, r_2/r, and so on. This process is reiterated for the duration desired. Figure 22.9 shows results of numerical simulations. Note the resemblance with real time series (compare with subplots of Figures 22.6 and 22.10).

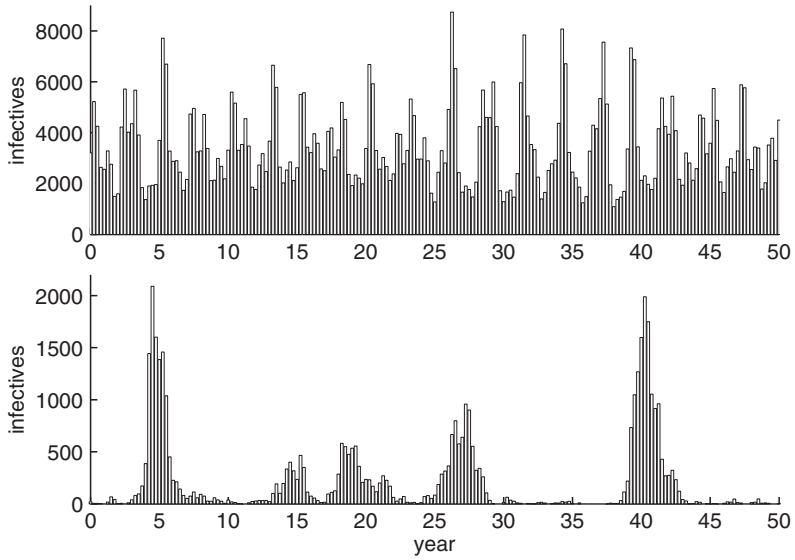


Fig. 22.9. Stochastic realizations of an *SIR* model in populations of 1,000,000 (top) and 100,000 (bottom) individuals. Each bar represents the incidence for one trimester.

22.8 DATA ANALYSIS

So far we have presented a variety of disease models, each with its advantages and disadvantages. It should be clear now that there is no one model which is, in absolute, better than the others. The best model depends on the question under investigation. This section is more oriented toward the epidemiological data. We will first see how model parameters can be estimated from the data and then focus specifically on the analysis of longitudinal data by presenting the basic tools of time series analysis.

22.8.1 Parameter Estimations

We have seen that mathematical models are characterized by a certain combination of parameters, each with a biological significance such as the force of infection, the birth rate, and so on. We have also seen that mathematical models allow the derivation of concepts which are not directly perceptible on the data, such as the basic reproduction number. In this section we are interested in trying to evaluate the numerical values of these quantities.

All the model parameters can be estimated by maximizing a model likelihood on real data. This procedure is largely used in modeling – not only in epidemiology – and its basic principles are presented in Box 22.2. Parameter estimation by maximization of the likelihood takes into account an error structure and thus allows giving a confidence interval on the estimation. This is one major advantage of the likelihood methods.

We will not present again the likelihood method here (see Box 22.2 for more details). Instead, we are interested in this section on the derivation of parameter values, almost from direct reading from the data, after playing a little bit with the model equations. Contrary to the likelihood methods, this method does not produce a confidence interval on the parameter estimation – though such an interval can be produced by Monte Carlo simulations (see Section 22.8.1.5). However, this method of parameter estimation is easy, direct, and much faster to implement than the likelihood methods.

22.8.1.1 The basic reproduction ratio R_0 In the previous sections we have seen two expressions of the basic reproduction number which can all help to estimate it from the data. The first one evidenced in Section 22.6.1 is relative to the endemic equilibrium value of the proportion of susceptibles in the population. Indeed, by searching the equilibrium point of the system of differential equations we ended with the fact that, at endemic equilibrium, the proportion of susceptibles in the population is equal to the inverse of the basic reproduction number. It is intuitively expected that the higher the basic reproduction number, the lower the proportion of susceptibles at endemic equilibrium in the population. A standard serological survey can easily determine the proportion s^* of susceptibles of an endemic disease. From this proportion one can thus determine the basic reproductive ratio simply as

$$R_0 = \frac{1}{s*} \tag{22.23}$$

Such estimations of the basic reproduction number of a variety of viral and bacterial infections are listed in Table 22.2. In Section 22.6.2 we showed an even simpler form of the basic reproduction ratio, provided we have the age of each case notification. From these data one can easily calculate the mean age at infection A. The life expectancy L is a demographic information that is available for many human populations. Dividing it by the mean age at infection produces a

TABLE 22.2. Some Disease Parameter Values Taken from the Literature. All Parameters are Estimated in Western Countries Unless Otherwise Specified

Diseases	γ^a	A^b	R_0^c	p_c^d	T_{obs}^e	T_{calc}^f
Measles	6–7	4–6[g], 1–3[h]	16–17	90–95%	1–2	1–2
Mumps	4–8	6–7	7–8[g], 11–14[h]	85–90%	3, 2–4	3, 2–4
Whooping cough	7–10	4–5	16–17	90–95%	3–4	3–4
Rubella	11–12	9–10[g], 2–3[h]	6–7[g], 15–16[h]	82–87%	3.5	4–5
Chickenpox	10–11	6–8	7–8[g], 10–12[h]	85–90%	2–4	3–4
Smallpox	—	—	—	70–80%	5	4–5
Malaria	—	—	—	99%	—	—

[a]Recovery rate (data from [10, 17, 25]).

[b]Mean age at infection (data form [5]).

[c]Basic reproduction ratio (data from [3, 5, 49]).

[d]Critical mass vaccination coverage [7].

[e]Observed interepidemic period (data from [5]).

[f]Model-predicted interepidemic period (data from [5]).

[g]Western countries.

[h]Developing countries.

good estimate of the basic reproduction ratio of a disease in a given population:

$$R_0 = \frac{L}{A} \tag{22.24}$$

Again this relation seems reasonable as it is intuitively expected that the higher the basic reproduction number R_0, the lower the mean age at infection A.

22.8.1.2 The force of infection λ In Section 22.6.2 too we arrived at the intuitively plausible conclusion that the mean age at infection is the reciprocal of the force of infection. Thus, knowing the age of the disease cases, one can easily calculate the mean age at infection A and deduce the force of infection:

$$\lambda = \frac{1}{A} \tag{22.25}$$

For the cases dealing with an age-structured model as in Section 22.7.2, we need to evaluate the force of infection by age cohort. By definition, the force of infection is the probability for a susceptible to get infected. As the events of disease transmission are independent, the number of susceptible follows negative exponential distribution of parameter equal to the force of infection.[2] Said in other words the ratio $S(a+1)/S(a)$ decreases exponentially at a rate equal to the force of infection:

$$\frac{S(a+1)}{S(a)} = \exp(-\lambda(a)) \tag{22.26}$$

[2]Incidentally, we find again Equation (22.25). Indeed the mean of a negative exponential distribution is, by definition, the inverse of the distribution parameter (see Box 22.3). Thus the mean age at infection is the reciprocal of the force of infection, as in Equation (22.25).

From Equation (22.26) the force of infection by age can thus be easily calculated as long as we are in the possession of disease prevalence $I(a)$ by age cohorts. Indeed, the number of susceptibles at age 0 is simply equal to the number of newborns ($S(0) = \mu N$) and the other values of $S(a)$ are then obtained recursively:

$$S(a+1) = S(a) - I(a) \tag{22.27}$$

22.8.1.3 The coefficient of transmission β In the case of airborne diseases, the force of infection λ is formally related to the coefficient of transmission β. The simplest of such relations is a linear one (see Section 22.5.1):

$$\lambda = \beta i \tag{22.28}$$

The coefficient of transmission β can thus be estimated from the value of the force of infection λ, as estimated in the above section 22.8.1.2, and the prevalence i.

For age-structured models, things become a little bit tougher. Indeed, from Equation (22.20) we have a system of n equations with n^2 unknown variables $\beta_{i,j}$:

$$\hat{\lambda}_i = \sum_{j=1}^{n} \hat{\beta}_{i,j}\hat{i}_j \qquad i = 1,\ldots,n \tag{22.29}$$

In order to solve this system, it is necessary to formulate hypotheses allowing us to decrease the number of unknown variables down to n. The first of these hypotheses is an assumption of symmetry [7]:

$$\hat{\beta}_{i,j} \equiv \hat{\beta}_{j,i}, \qquad (i,j) \in \{1,\ldots,n\}^2 \tag{22.30}$$

However, this hypothesis has the effect of decreasing the number of unknown variables only to $n(n+1)/2$. An alternative

consists in defining $\hat{\beta}_{i,j} \equiv \hat{\beta}_i$ [7]. In any case these hypotheses necessitate the definition of a WAIFW (who acquire the infection from whom) matrix. Considering $n = 5$ age cohorts, the four most usually used WAIFW matrices are the followings [7]:

$$\text{WAIFW}_1 = \begin{bmatrix} \beta_1 & \beta_1 & \beta_3 & \beta_4 & \beta_5 \\ \beta_1 & \beta_2 & \beta_3 & \beta_4 & \beta_5 \\ \beta_3 & \beta_3 & \beta_3 & \beta_4 & \beta_5 \\ \beta_4 & \beta_4 & \beta_4 & \beta_4 & \beta_5 \\ \beta_5 & \beta_5 & \beta_5 & \beta_5 & \beta_5 \end{bmatrix}$$

$$\text{WAIFW}_2 = \begin{bmatrix} \beta_1 & \beta_1 & \beta_1 & \beta_4 & \beta_5 \\ \beta_1 & \beta_2 & \beta_3 & \beta_4 & \beta_5 \\ \beta_1 & \beta_3 & \beta_3 & \beta_4 & \beta_5 \\ \beta_4 & \beta_4 & \beta_4 & \beta_4 & \beta_5 \\ \beta_5 & \beta_5 & \beta_5 & \beta_5 & \beta_5 \end{bmatrix}$$

$$\text{WAIFW}_3 = \begin{bmatrix} \beta_1 & \beta_1 & \beta_1 & \beta_1 & \beta_1 \\ \beta_2 & \beta_2 & \beta_2 & \beta_2 & \beta_2 \\ \beta_3 & \beta_3 & \beta_3 & \beta_3 & \beta_3 \\ \beta_4 & \beta_4 & \beta_4 & \beta_4 & \beta_4 \\ \beta_5 & \beta_5 & \beta_5 & \beta_5 & \beta_5 \end{bmatrix}$$

$$\text{WAIFW}_4 = \begin{bmatrix} \beta_1 & \beta_5 & \beta_5 & \beta_5 & \beta_5 \\ \beta_5 & \beta_2 & \beta_5 & \beta_5 & \beta_5 \\ \beta_5 & \beta_5 & \beta_3 & \beta_5 & \beta_5 \\ \beta_5 & \beta_5 & \beta_5 & \beta_4 & \beta_5 \\ \beta_5 & \beta_5 & \beta_5 & \beta_5 & \beta_5 \end{bmatrix}$$

where classes 1, 2, 3, 4, and 5 usually refer to the 0–4 years, 5–9 years, 10–14 years, 15–19 years and 20 years more, respectively. Each of those matrices yields a system of n equations with n unknown. The $\hat{\beta}_i$ can thus be determined from the observed mean incidences by age cohort (i) and the mean forces of infection by age cohort calculated in the above Section 22.8.1.2.

For a temporally varying coefficient of transmission, there is no other means of estimation than maximum likelihood, whatever the exact form of the variation on β.

22.8.1.4 The rate of recovery γ Quite generally, under steady-state conditions, the quantity in a given compartment is equal to the product of the rate of inflow times the expected sojourn time. In the case of the infective compartment of an *SIR* model this remark translates into *incidence* × *expected sojourn time* = *prevalence* [19]. As mentioned in Section 22.6.1, when the outflow of a compartment occurs at a constant rate, the sojourn time in the compartment follows a negative exponential distribution with parameter equal to the rate of outflow. The mean of a negative exponential distribution is equal to the reciprocal of its parameter, thus the expected sojourn time in the infective compartment is equal to the inverse of the recovery rate. In consequence, we end up with the following:

$$\gamma = \frac{incidence}{prevalence} \tag{22.31}$$

Epidemiological data generally contain either incidence or prevalence. However, one can be easily calculated form the other as incidence is equal to the variation of prevalence.

22.8.1.5 Monte Carlo simulations We have presented here simple methods to estimate the values of both model parameters (such as the rate of recovery) and emerging quantities (such as the basic reproduction number). These estimations are fast and easy to implement with most available epidemiological data. However, and contrary to likelihood methods, they do not provide any confidence interval on the estimation. One classic method to cope with this is to use Monte Carlo simulations.

The idea of Monte Carlo simulations is to generate a distribution of a parameter by resampling the data [45]. A confidence interval can then be found based on this distribution. In practice, the generation of such a distribution is done as follows. (i) an artificial data of the same length as the original data set is generated by sampling with replacement in the original data set. (ii) The parameter is estimated on this artificial data set and its value kept in memory. Steps (i) and (ii) are repeated a large number of times and the values of the parameters estimated on each artificial data set give a distribution of the parameter. From this distribution one can find a confidence interval. One crucial point in Monte Carlo simulations is related to the choice of the number of time steps (i) and (ii) should be repeated. This number should be large enough for the generated parameter distribution to be considered in a steady state. One way to check for the convergence of the distribution toward a steady state is to follow the evolution of the value of one distribution's statistic (such as the mean) at each new repetition and stop when this statistic seems to have converged to a steady value.

22.8.2 Tools for Time Series Analysis

Longitudinal epidemiological surveys produce time series. The object of time series analysis is to look for periodic patterns in the data. Because of the time component, data in time series are not independent. The consequence is that the classic statistical tools that assume independence of data cannot be used on time series [15]. In this section we briefly present the basic tools of time series analysis, from the simplest to the most recent and elaborated.

22.8.2.1 Stationary time series The first two methods require the time series to be in a stationary state. A time series is said to be in a stationary state if there is no systematic change in mean (no trend) and in variance [15]. This basically supposes that the signal has constant period and amplitude, which is not the case for numerous real time series. The trend can be removed by considering the residuals from a regression or a nonparametric smoothing such as a B-spline or Loess regression [15]. Another mean for removing the trend consists in applying a moving average to the series [15]: each point a time t in the series is replaced by the average of the points between

times $t - T/2$ and $t + T/2$, where the length T of the moving window is to be defined. When T increases the edge effects increase too and the averaged series will be reduced from T data points compared to the original series. Lastly, the trends can be removed by considering the variations in the time series [15]: each data point is replaced by the difference with the previous data point. Square transformation of the data generally has the effect of stabilizing the variance and logarithm transformation is usually used to linearize the data, the three methods presented here requiring linear data sets where the effect is proportional to the cause.

22.8.2.2 Autocorrelograms This method applies to stationary time series. The idea is to calculate the correlation between a time series and a lagged copy of itself [15] (Figure 22.10). The autocorrelogram plots the value of an autocorrelation coefficient r against the value of the lag. At lag = 0 the autocorrelation is by definition equal to 1. When the lag increases the value of the autocorrelation coefficient decreases, then becomes negative, and oscillates around the $r = 0$ horizontal line. The period of the oscillations of the periodogram is the same as the period of the original signal. Moreover, the oscillations are damped. Indeed, because of the dependency between the points of the time series, the noise accumulate additionally, thus hiding any autocorrelation signal when the lag becomes too large. In the same way one can trace correlograms between two different series (called cross-correlograms) to get an idea of the synchronicity or phase difference between two data sets.

22.8.2.3 Fourier spectra Like the autocorrelogram this method applies to stationary time series. The Fourier theorem states that any periodic signal $s(t)$ of frequency F_0 can be decomposed into a sum of sinusoids [13, 15] (Fig. 22.11):

$$s(t) = a_0 + \sum_{n=1}^{+\infty} \left[a_n \cos(2\pi F_0 nt) + b_n \sin(2\pi F_0 nt) \right] \quad (22.32)$$

where a_0 is the mean of the signal and a_n and b_n ($n \in \mathbb{N}$) are the Fourier coefficients, basically referring to the weight that each harmonic of frequency nF_0 has in the whole signal. This can also be thought of in terms of the magnitude of the correlation between the signal $s(t)$ and the sinusoid of frequency nF_0. The use of complex numbers renders this formula much simpler[3]:

$$s(t) = \sum_{n=-\infty}^{+\infty} c_n e^{jn(2\pi F_0)t} \quad (22.33)$$

where j is the imaginary number and the Fourier coefficients c_n are now complex. The advantage of this form is that we have only one coefficient c_n for each frequency nF_0. Decomposing a time series into a Fourier sum consists in estimating the coefficients of the Fourier sum. For Equation (22.33) the coefficients are equal to

$$c_n = \frac{1}{T_0} \int_0^{T_0} s(t) e^{-j2\pi F_0 nt} dt \quad (22.34)$$

It is important to realize that the time series $s(t)$ and the series of Fourier coefficients c_n describe exactly the same reality, the time series in the time domain and the Fourier coefficients in the frequency domain. The Fourier transform of Equation (22.34) allows passing from the time to the frequency domains whereas the reverse Fourier transform of Equation (22.33) does the opposite transformation. The time or the frequency domains are thus two different ways of looking at the same reality. As in time series analysis we are interested into the regular patterns of a series, it is often more convenient to work into the frequency domain (at least when the series are stationary). This decomposition of a periodic signal into a sum of sinusoids can be generalized to the decomposition of an aperiodic signal where an aperiodic signal is simply a periodic signal with a period equal to ∞ [13,15]. Equation (22.34) then takes the more general form

$$S(f) = \int_{-\infty}^{+\infty} s(t) e^{-j2\pi ft} dt \quad (22.35)$$

where the Fourier transform $S(t)$ is now a continuous function of frequency f. A Fourier spectrum plots the values of $S(f)$ against f. Inspection of such a spectrum gives a clear idea of which frequencies contribute the most to the signal.

22.8.2.4 Wavelet analysis Direct and inverse Fourier transforms force us to visualize a stationary time series either in the time or the frequency domain. Analyzing nonstationary time series, one may, however, be interested in visualizing it in the time and frequency domain at the same time in order to be able to say that the period of the signal is equal to T_1 between times t_1 and t_2; T_2 between times t_2 and t_3; and so on. One first attempt into this direction has been the use of the Fourier transform on a moving window. The major disadvantage of this *ad hoc* method is that the fixed size of the windows gives different weights to the different frequencies. This inconvenience has been coped by the invention of the wavelets.

The last decade has witnessed the emergence of an impressive number of wavelets. Certainly, the most used in ecology is the Morlet one which is essentially a complex exponential with a Gaussian envelope. The key advantage of wavelets relative to sinusoids used in Fourier analysis is that not only they can be moved along the signal (as in windowed Fourier analysis) but also they can be stretched to account equally for the different frequencies [13,58]. A wavelet spectrum is thus a three-dimensional graph which plots the correlation of the wavelet with the signal as a function of both the location of the wavelet along the signal (time domain) and the stretching of the wavelet (frequency domain). Figure 22.12 shows an example for the measles

[3] After the application of the key mathematical relationship: $e^{j\theta} = \cos\theta + j \sin\theta$.

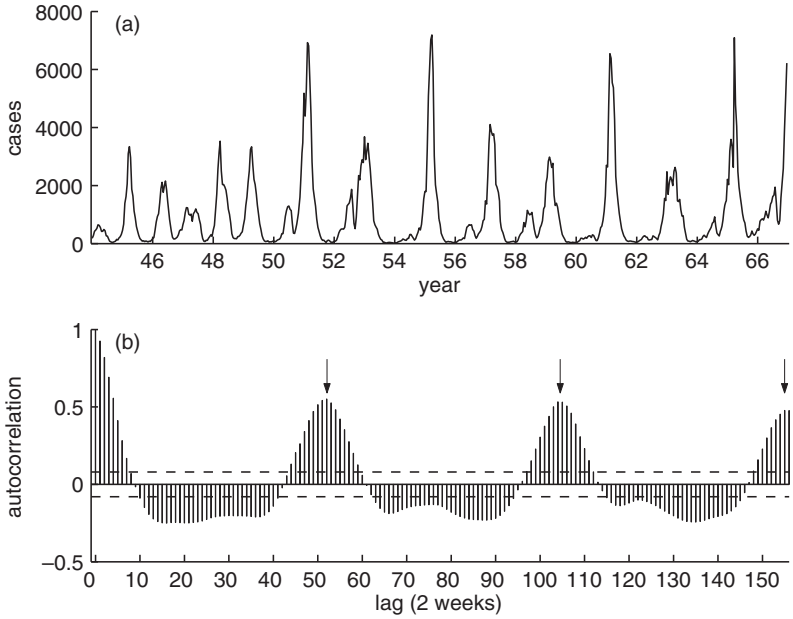


Fig. 22.10. Autocorrelation plot of the biweekly measles notification cases for London from 1944 to 1966. (a) Time series of the cases. The correlation between the time series and a lagged copy of itself is calculated. As the notifications are biweekly, the lag is equal to 2 weeks. (b) The autocorrelogram shows the value of the autocorrelation against the lag. The dashed line represent the 95% confidence limits about zero. When lag = 0 the series is correlated with itself and thus the autocorrelation is equal to 1. The autocorrelation coefficient then reaches maxima at lag = 53, 105, and 156 (106, 210, 312 weeks, respectively, see vertical arrows), thus evidencing a period of about 2 years. Data downloaded from http://www.zoo.cam.ac.uk/zoostaff/grenfell/measles.htm [27].

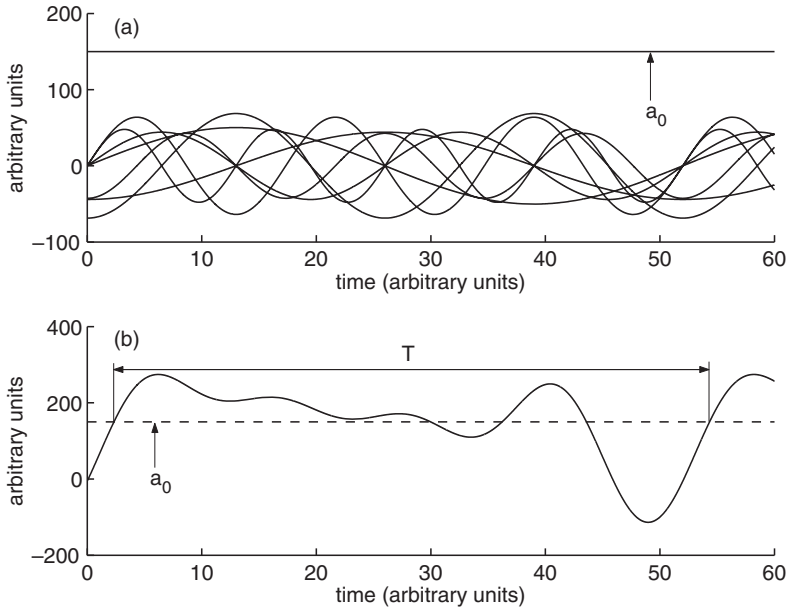


Fig. 22.11. Decomposition of a periodic signal into Fourier sum of sinusoids. (a) Plot of the sinusoidal component of the whole signal (b). With reference to Equation (22.32) $a_0 = 150$, $a_1 = 244.16$, $b_1 = 20.22$, $a_2 = 268.66$, $b_2 = 44.22$, $a_3 = 242.34$, $b_3 = 63.82$, $a_4 = 0$, $b_4 = 47.83$, $T = 1/F_0 = 52$ arbitrary units.

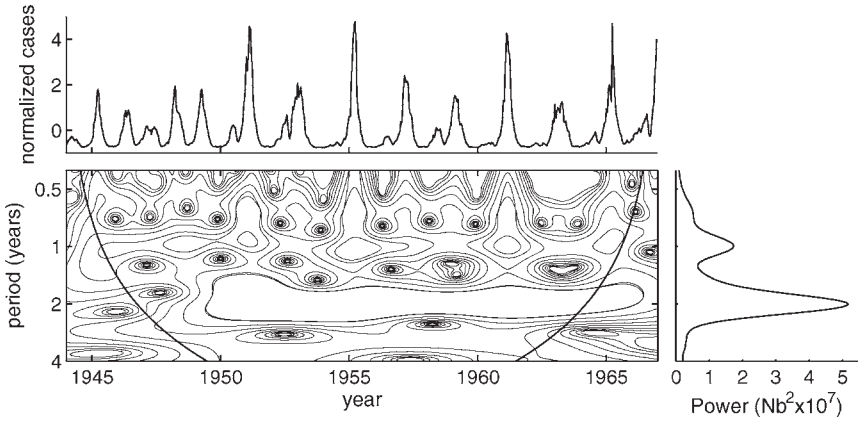


Fig. 22.12. Wavelet power spectrum of the measles notification cases for London between 1944 and 1966. The magnitude of the correlation between the series and the wavelet increases from blue to black. The parabolic curves represent the cone of influence. Because of edge effects, everything below the cone of influence cannot be interpreted. The above graph shows the time series of normalized measles cases and the right graph is the Fourier spectrum. It is clear that the wavelet power spectrum combines information of both time and frequency domains. Data downloaded from http://www.zoo.cam.ac.uk/zoostaff/grenfell/measles.htm [27]. See color plates.

cases of London from 1944 to 1966. With such a graph one is able to say the frequency of a signal at any time.

22.9 APPLICATIONS TO VACCINATION POLICIES

After an overview of the basic epidemiological models and results, as well as statistical tools for the epidemiologist, the last section is devoted to practical applications for the development of vaccination policies. In public health, vaccination policies are decisions made by governments and applied on large spatial and temporal scales. The ultimate aim of a vaccination policy is the eradication of a disease from a population. This goal is extremely difficult to achieve in practice and most vaccination strategies are imperfect in the sense that they only decrease (sometime dramatically) the number of cases, without, however, eradicating the disease [7]. In this context, vaccination can yield side effects on the disease statics and dynamics that are important to evaluate. There currently exist two major vaccination strategies – the mass vaccination, which is the most ancient and still the most applied, and the recently developed pulse vaccination which is used in a increasing number of countries.

22.9.1 Mass Vaccination Strategy

Mass vaccination strategy is the most ancient and still the most widely used vaccination scheme. It consists in vaccinating a large proportion of infants before the mean age at infection [7], for example, the 0–2 age cohort for the measles-mumps-rubella (MMR) vaccine in the United States. Its first applications started in the sixties against measles in the North American and European countries where they have caused a dramatic decrease of the number of cases.

22.9.1.1 Calculating the vaccination coverage The derivation of the optimal vaccination coverage is based on the properties of the endemic equilibrium. At equilibrium the replacement number R (see Section 22.5.3) is equal to the basic reproduction number R_0 times the proportion of susceptibles: $R = R_0 s^*$. Applying a vaccination coverage equal to p has the effect of diminishing the proportion of susceptibles by p: $s^* = 1 - p$. A condition for disease eradication is that the reproduction number be less than 1: $R = R_0 s^* = R_0(1 - p) < 1$, or $p > 1 - 1/R_0$. Thus the critical vaccination coverage p_c for disease eradication is $p_c = 1 - 1/R_0$ [7]. Note that this result shows that we do not need to vaccinate each individual to protect the whole population. Note too that this property known as herd immunity is not evident from the data and emerges only from the model. The higher the basic reproduction number, the higher the vaccination coverage should be. Vaccination coverages of major human infectious diseases are given in Table 22.2. We can see that many infectious diseases require vaccination coverage which are far too higher to be achieved in practice. This is further complicated by other mechanisms such as the vaccine efficacy. Consider, for example, measles and rubella for which estimates of the critical vaccination coverage based on R_0 are 0.94 and 0.86, respectively. A vaccine efficacy of 0.95 means that 5% of those vaccinated do not become immune. In consequence, taking into account vaccine efficacy necessitates coverages of 0.99 and 0.91 for measles and rubella, respectively [34]. This explains why the only human infectious disease which has been eradicated successfully worldwide so far is smallpox which has the lowest critical vaccination coverage.

22.9.1.2 Consequences on the statics A first and expected effect of vaccination is that fewer people will

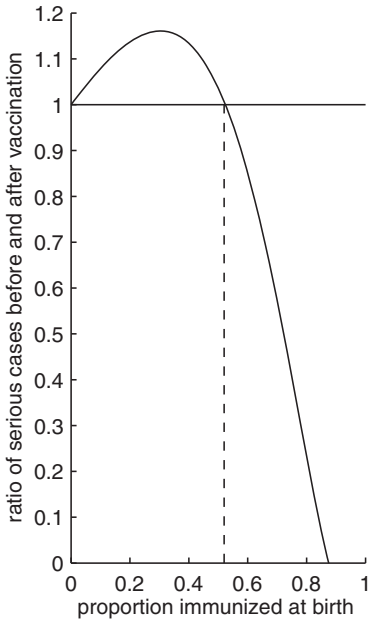


Fig. 22.13. Model predicted effect of a mass vaccination policy at birth on the number of congenital rubella syndromes (CRS). The graph plots the ratio of CRS before and after the start of the mass vaccination policy against the vaccination coverage. It shows that low vaccination coverages (less than 50% here) should be avoided as they can increase the absolute number of serious cases (model from [4]).

experience infection. But the decrease of the force of infection due to vaccination means that the mean age at infection of the smaller number of people who do acquire infection increases [7] (recall Equation (22.25)). If the probability of disease complications increases with age, it is thus possible that some vaccination programs could actually increase the absolute number of serious cases. The likelihood of such a perverse outcome again can only be evaluated thanks to disease models. A classical example is the one of the congenital rubella syndrome (CRS), treated in detail by Anderson and May [4]. They have evidenced that the absolute number of CRS can actually increase with the vaccination coverage when the vaccination coverage is low (Figure 22.13).

22.9.1.3 Consequences on the spatial and/or temporal dynamics The spatio-temporal dynamics of a disease is of primary interest if we are to design efficient country-wide vaccination policies [28]. For example, the global eradication of a disease would be easier if the local dynamics are synchronous. In the case where local dynamics are completely asynchronous, local extinctions would be quickly followed by migration of infectious individuals from neighbor communities experiencing an epidemic outbreak. With a very simple endemo-epidemic model Earn et al. [22] have shown that the disease dynamics complexity – as given by the length of the period and the number of different attractors (see Box 22.4) – increases with vaccination coverage. Furthermore, a simple island model such as the one presented in Section 22.7.3 evidences that an increase in the vaccination coverage results in a decrease of the spatial synchrony of disease dynamics. This is intuitive as the coupling between the different subpopulations is assured by the migration of infective (see Equation (22.21)). Vaccination has the effect of decreasing the number of infectives and thus the synchrony between the different subpopulations. In conclusion, mass vaccination has the effect of (i) increasing disease dynamics complexity and (ii) desynchronizing local dynamics. The first consequence accentuates the second as the probability of dynamics synchronicity naturally decreases with the level of complexity. These model predictions have been successfully confirmed on real data analysis [52]. As the mass vaccination tends to desynchronize the spatial dynamics of the disease, it renders global disease eradication even more difficult to achieve in practice than expected from the above theoretical predictions (Section 22.9.1.1).

22.9.2 Pulse Vaccination Strategy

As highlighted in the previous Section 22.9.1, mass vaccination strategy requires a too high systematic vaccination coverage to be achieved in practice. A recently proposed and potentially less expensive strategy is vaccination in pulses [1, 50]. This approach consists in vaccinating a certain proportion of the population at regular intervals of time. The rationale behind this is to vaccinate sufficiently and frequently enough to maintain the percentage of susceptibles below the threshold necessary for an epidemic to start (Figure 22.14). What makes this policy less expensive than the mass vaccination strategy is that it explicitly accounts for the dynamics of the host population through the birth rate. Several theoretical works have been carried out to express the optimal vaccination coverage and frequency as a function of the host demographic characteristics [21,55,57]. The simplest one is derived from the Pythagore theorem (see Figure 22.14). This theory has been successfully applied in campaigns against poliomyelitis and measles in Central and South America, and measles in the United Kingdom in 1994.

22.9.2.1 Spatial dynamics Using a simple endemo-epidemic model Earn et al. [23] have shown that a same pulse vaccination strategy tends to synchronize disease dynamics in independent localities (see Figure 22.15). This phenomenon is a case of Moran effect where the same causes produce the same effects [12, 30, 47]. Indeed, by its periodical nature, an imperfect pulse vaccination strategy acts as a forcing driving the disease dynamics. Two independent populations submitted to the same pulse vaccination scheme will thus exhibit similar and synchronous disease dynamics. The effect of pulse vaccination on the spatial dynamics of a disease is thus opposite to the one of mass vaccination and this facilitates the achievement of a global disease eradication [38].

22.9.2.2 Resonance A second side-effect that can be associated to the periodic nature of pulse vaccination is the phenomenon of resonance. Theory of oscillator dynamics predicts

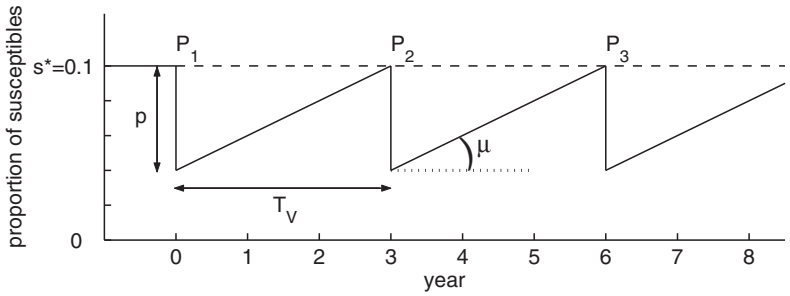


Fig. 22.14. Pulse vaccination scheme. The graph shows the proportion of susceptibles as a function of time. The aim is to find a vaccination coverage p and a frequency $1/T_V$ of the vaccination pulses P_1, P_2, P_3, and so on, such as the proportion of susceptibles stays below the critical value s^* necessary for an epidemic to start (represented by the dashed line). Births fill the stock of susceptibles at a constant rate μ.

that an oscillating dynamical system (such as an epidemiological one) submitted to a periodic forcing (such as an imperfect pulse vaccination) can produce phenomena of resonance [36]. Resonance is a generic term indicating that the amplitude of observed oscillations depends on the period of the forcing, and has a maximum, called peak of resonance. These theoretical predictions and their epidemiological consequences have been investigated on a disease system by numerical simulations and data analyses [16]. Figure 22.16 shows that the mean annual number of infectives globally decreases as the frequency of vaccination pulses increases. However, resonance is responsible for the peaks observed on this general trend. The major one occurs at a vaccination frequency close to 2 years, the others simply being harmonics of it. The practical consequence of these peaks is that, locally on the vaccination frequency dimension, the mean annual number of infectives counter-intuitively increases with the frequency of vaccination.

22.10 CONCLUSION

22.10.1 What We Have Seen

Statistical analyses of epidemiological data help to characterize, quantify, and summarize the way diseases spread in host populations (Section 22.8). The aim of epidemiological modeling is to understand the behavior of diseases in nature (Section 22.2). Because of ethical and practical impossibility to perform experiments in public health, mathematical models appear as a cheap and efficient way to explore and test hypotheses (Section 22.2.2). In addition to force the investigator to think rigourously (Sections 22.5.1 and 22.5.2, Box 22.3), models provide powerful conceptual results such as the basic reproduction number and threshold effects (Section 22.5.3), or the herd immunity (Section 22.9.1.1). Even if very interesting pure theoretical works have been realized, the key element of epidemiological modeling is to

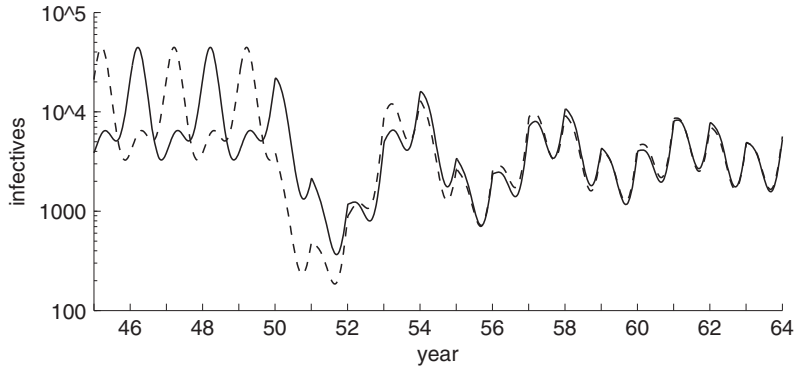


Fig. 22.15. Effect of a same pulse vaccination strategy on independent disease dynamics. The simulations start so that the disease dynamics are in opposition of phase. At year 50 a pulse vaccination strategy is started with $p = 20\%$ and $T_V = 1$ year, progressively synchronizing the two dynamics.

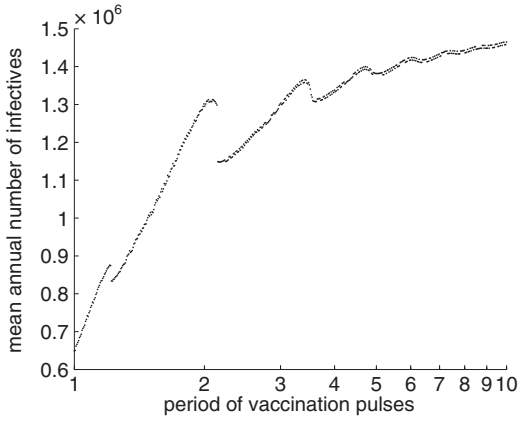


Fig. 22.16. Mean annual number of infectives as a function of the period T_V of vaccination pulses. The general trend is a decrease of the mean annual number of infectives as the frequency of vaccination ($1/T_V$) increases. However, resonance is responsible for these peaks on this general trend. The consequence of such peaks is that, locally on the vaccination frequency dimension, the mean annual number of infectives counter-intuitively increases with the frequency of vaccination.

link model with data. Likelihood methods are modern and efficient ways to do so (Box 22.2). Models thus allow to estimate epidemiological parameters and also to identify crucial data that need to be collected.

This chapter was centered on the *SIR* model. Although one of the simplest epidemiological models, it is still one of the most used, particularly to study childhood viral and bacterial infections. There is a multitude of ways to complexify this simple model in order to account for more and more phenomena. However, the more complex is not necessary the best and it is the purpose of a model that dictates its degree of complexity (Section 22.2, Box 22.1). We have explored models to study single epidemics (Section 22.5), endemic diseases (Section 22.6), spatial disease dynamics (Section 22.7.3), and have illustrated how these mathematical tools can be used for the development of public health policies in helping defining optimal vaccination strategies (Section 22.9).

22.10.2 What We Have Not Seen

Of course, the list of what we have not seen about epidemiological models is much longer than the list of what we have glanced at.

Some classes can be added to the simple *SIR* model. For example, a commonly used model for childhood diseases is the *SEIR* one which adds a class of exposed (E) individuals, that is, individuals which are infected but not infectious yet. For most childhood infectious diseases the latence phase is often as long as the infectious and should be accounted for as it can substantially change the epidemiological conclusions of the models. *MSEIR* models further add a class accounting for the post-birth period during which newborns are protected from infections by maternal antibodies.

All the models covered in this chapter assume that the host population is of constant size. This, thus, excludes both diseases in exponentially growing populations like in most developing countries and disease-induced mortality as the case for many infections including childhood diseases in developing countries, malaria, and so on. Accounting for a nonconstant host population size requires the explicit modeling of the host population dynamics, in addition to the disease dynamics.

For sexually transmitted diseases (STD), contagious contacts are not established randomly as usually assumed for airborne infections. Besides a strong heterogeneity in the sexual activity, sexual contact occurs preferentially between people of the same sexual activity, creating this core effect in the epidemiology. Models for STD should thus account for all these degrees of heterogeneity in the contacts. In addition, many STD result in little or no acquired immunity following recovery, and *SIRS* models would be more appropriate.

Other particular epidemiological systems requiring adapted models include, among others, mother-to-child diseases for which not all children are born into the susceptible compartment and diseases propagated by syringe-sharing intravenous drug users such as HIV/AIDS.

The epidemiology of multiple-host diseases is far more complicated than the one of directly transmitted infections. Compartmental models of such diseases need to account for the dynamics of the disease in the different hosts or reservoirs, and possibly also for the dynamics of these different hosts or reservoirs. For these diseases, the modeling of passage from one host to the other is not always an easy task.

Lastly, so far we have dealt primarily we microparasitic infections. Contrary to microparasites (Section 22.4), macroparasites refer to large-size parasites (helminths, arthropods) with direct reproduction in the definitive host. Macroparasites generally have longer generation time (often an appreciable proportion of the host life span) than microparasites. The immunity following the recovery from a macroparasitic infection is generally of short duration and the number of parasites per host is a strong determinant of the epidemiology. From a modeling point of view, this means that the simple compartmental models presented in this chapter for microparasites should be replaced by more complicated models accounting for the distribution of parasites among the hosts. This is totally another subject and we refer the reader to the classical book by Anderson and May [7] for more details.

22.11 SUMMARY

By clarifying rigorously the assumptions, the variables, and the parameters, mathematical modeling allows understanding the observed spread of diseases in space and time. Epidemiological model further provides important conceptual results including the basic reproduction number, the threshold effects, and the herd immunity. For evident ethical and practical reasons, experiments in public health are often impossible to perform and mathematical models thus appear as a cheap and efficient

way to explore and test hypotheses. This is, for example, of particular practical utility in the design of vaccination policies. One key aspect of epidemiological models is their link to real data. Such data often stand under the form of time series which necessitate specific statistical tools for their analysis. Models can always be complicated to improve their fit to real data. However, more complex models are not always the best and it is the question under investigation that should dictate the optimal level of complexity.

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CHAPTER 23

Using a Geographic Information System to Spatially Investigate Infectious Disease

A. Curtis,[1,2] J.K. Blackburn,[3] and Y. Sansyzbayev[4]

[1]*World Health Organization Collaborating Center for Remote Sensing and GIS for Public Health, Louisiana State University, Baton Rouge, LA 70803*

[2]*Department of Geography and Anthropology, Louisiana State University, Baton Rouge, LA 70803*

[3]*Department of Geography, California State University, Fullerton, 800 N. St. College Blvd., Fullerton, CA 92834-6846*

[4]*Technology Management Company, 29/6 Akademik Satpaev Ave., Business Tower, 5th floor, HYATT Regency Hotel, Almaty, Kazakhstan*

23.1 INTRODUCTION

The geographic investigation of an infectious disease often involves a search for spatial patterns of cases, followed by the association of other geographic or environmental phenomena that coincide with those patterns. Once these exploratory analyses have been performed, hypotheses can be tested as to how geographic space influences, and sometimes promotes, disease presence. A *geographic information system* (GIS) is a tool that readily facilitates this type of spatial epidemiological approach. This chapter will provide a basic overview of how this technology is used in epidemiological investigations. The examples here draw heavily from current projects at the *World Health Organization Collaborating Center for Remote Sensing and GIS for Public Health* (WHOCC) at Louisiana State University, and include the recent GIS support the Center provided during Hurricane Katrina. Throughout this chapter examples will be presented to illustrate the similarities between the GIS tools for infectious disease studies and emergency response mapping. This chapter should be seen as an accompaniment to other discussions in this book concerning the allied technology of remote sensing.

Why is a spatial approach important? Consider the scenario of an emerging disease of unknown etiology being reported. By collecting as much spatial data as possible, such as case locations, underlying vegetation type, soil moisture, and anthropogenic variables, an exploratory spatial analysis can be performed to identify geographic patterns of disease diffusion and associations between cases and environmental conditions. This approach can help in the identification of a possible vector, or in determining the ecological landscape of the disease. For a reasonably well-understood pathogen, spatial analysis can be used to predict disease occurrence in both space and time, either through diffusion modeling, or by finding associations between different geographic layers. If the geography of the disease is well understood, then the spatial investigation can move beyond prediction and into prevention via changes in living practice or vaccine strategies[1]. Of course, in reality, diseases are not as discrete as this scenario; rather, a continuum exists between identifying patterns on the landscape and developing a comprehensive space–time understanding of the pathogen.

The geography of a disease can range from relatively simple to complex. For example, a simple disease transmission, such as smallpox – a deadly viral disease of the genus *Orthopoxvirus* – would generally only involve human settlement patterns and transportation corridors, whereas a disease with a complex geography could involve multiple environments, a vector, multiple hosts, microclimates, specific anthropogenic characteristics, explicit transportation corridors, and so on. This complexity makes Lyme disease one of the more popular diseases analyzed spatially within a GIS environment [14,23,30]. Lyme disease is caused by a bacterial infection from *Borrelia burgdorferi* and is vectored by ticks. For example, the tick vector in the Northeast, *Ixodes*

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

scapularis, prefers different blood meals during each of its three life stages. Each of these blood meals (e.g., the white-footed mouse and the white-tailed deer) has defined environmental preferences. The questing of the tick is also influenced by factors that create geographic patterns, including micro- and macromoisture and temperature, vegetation type, and even anthropogenic influences on suburban garden type. Overlaid onto these geographies are the diffusion pathways of both deer and migratory birds, which both redistribute ticks seasonally within endemic areas and introduce infected ticks into environmentally suitable but disease-free areas. To synthesize all of this spatial information would be difficult without the use of a GIS.

23.1.1 What Is a GIS?

Geographic information science (GISc) combines new geographic technologies, such as remote sensing and the global positioning system (GPS), along with advances in spatial analytical inquiry (finding patterns - through spatial hypothesis testing) and cartographic visualization (making maps and cartographic animations). The GIS is the technology/software that ties these advances together. For a more comprehensive overview of GIS the reader is pointed to an introductory GIS text [39]. In addition, several journal articles and collected works have been written that detail public health and epidemiological investigations using a GIS [4,5,31,52,62]. For the purpose of this chapter, a simple definition of a GIS is that it is a tool comprised of computer hardware, specific software applications, and a skill set that allows for the interactive storage, manipulation, analysis, and visualization of primarily spatial data. Although today GIS and geospatial analyses utilize high-speed computer technology, the usefulness of the approach can be illustrated with a historical example. In 1853, John Snow used what was in effect a pin map or dot map to reveal the source of a cholera outbreak in London. This analysis, if performed today, could easily have been achieved using a GIS. The origin of the cholera outbreak, calculated in several ways using spatial measures of central tendency found in many GIS packages, would have revealed a *hotspot* (an area of significant case clustering) containing the source of infection. In 1853 that calculation would have been visual, with the Broad Street pump being visually noted as the closest water source to the center of the outbreak. In actuality this historic piece of spatial epidemiology did not quite happen in this manner, with the map now believed to be a visual aid explaining the logic of John Snow's choice after the fact [43]. The same investigation today would include the *heads-up digitizing* of the cases, before a density is calculated to find the center of highest disease concentration (see section below on heads-up digitizing). Where the GIS approach vastly improves on spatial epidemiology of the 1800s is that additional GIS layers, such as housing values, names of the infected, or dates of infection can be *overlaid* (stacking data layers within the onscreen map environment) with a series of simple commands.

There are two basic GIS data formats: *vector* and *raster*. The main difference between these two formats is how the geographic data values are handled within a database. Vector GIS defines geography into three major objects: points (e.g., a disease case or an address), lines (e.g., the road connecting villages, a stream, a railroad line), and polygons (e.g., a political boundary, a building footprint, a water body). Descriptive data for each point, line, or polygon are stored as attributes in an accompanying data table that links each vector geometry with appropriate data values – *attributes*. A raster GIS uses a spatial grid with symmetrical cells to store the data. With raster data, attributes are assigned to each grid cell in the database and numerical values are used to represent various features. In this way, a variety of spatial layers, such as soil, vegetation, and elevation could all be recorded as different codes for a single cell. As each cell occupies the same space, multiple spatial layers can be compared within each cell. In this way raster GIS is useful for layering multiple environmental datasets and finding associations between them. Vector GIS is well suited for identifying clusters and diffusion patterns in a disease surface (preferably using point geometry).

As with any epidemiological investigation, reliable data are required. For input into a GIS these data must contain a spatial location. This location could be a coordinate system, a surveillance collection grid, a street address, or data aggregated to a political areal unit (such as a zip code, county, or province). The precision of these spatial locations is usually tied to whether the disease is primarily urban or rural, or occurs in a developed or developing country. Within urban environments, disease data are often available as spatially precise points, usually as a residential street address. Within rural environments data are often only recorded at the resolution of a large tract of land. Similarly, in developed countries technological availability and the presence of an established data infrastructure often result in more spatially precise data than in developing countries. In general, the more precision in the spatial data set, the greater the possibilities for an in-depth, analytical spatial investigation.

A further advantage of investigations conducted in urban environments of developed countries is that many secondary data sets are often available. These can be tied to disease case locations to either explain or predict the underlying spatial pattern. Secondary data sets are often extracted from a census, with fields such as average age, income level, or education providing insight into why the disease occurs in certain neighborhoods. For some locations, even more detailed census data exist, such as the percentage of children living in poverty, with "children" being further categorized into age and race cohorts. The GIS can be used to combine these social data layers with actual residences (spatial locations). This GIS manipulation is known as a point-in-polygon transformation, whereby victim "A" residing in census block "X" is given the attributes (data fields) of that census block. For example, Figure 23.1 (insets a and b) displays two maps generated after Hurricane Katrina, which made landfall along

the U.S. Gulf Coast on August 29, 2005. Figure 23.1(a) displays a measure of poverty by census block group (based on percentage of welfare recipients by polygon), and Figure 23.1(b) displays floodwater depths within New Orleans after the city's levee system failed due to the severity of the storm surge. The overlying point file identifies the location of storm-related deaths (randomized in this publication to protect victim confidentiality). The point-in-polygon transformation would allow a summary table to be created showing the distribution of deaths to each flood depth, or the number of deaths at each poverty level. This would allow questions to be answered such as: (i) *What was the association between flood depths and mortality locations* or (ii) *How many died in census block groups with more than 75% of residents claiming welfare?* The transformation itself works by attaching the flood depth of the grid cell, or the poverty level of the census block group, to each death location according to its spatial location on the map.

Other secondary data sets may include physical information about the neighborhood, such as the density of residences and shared living spaces (taken from large scale maps containing building footprints), or transportation corridors and key locations (such as health clinics or landfills). These data might be of importance as the disease under investigation may be related to overcrowding, to poverty, or to proximity to a polluted water supply. Unlike with the Hurricane Katrina example, the points (disease cases) might be analyzed to identify spatial patterns. If any *hotspots* are found, the secondary data layers might be used to identify the spatial

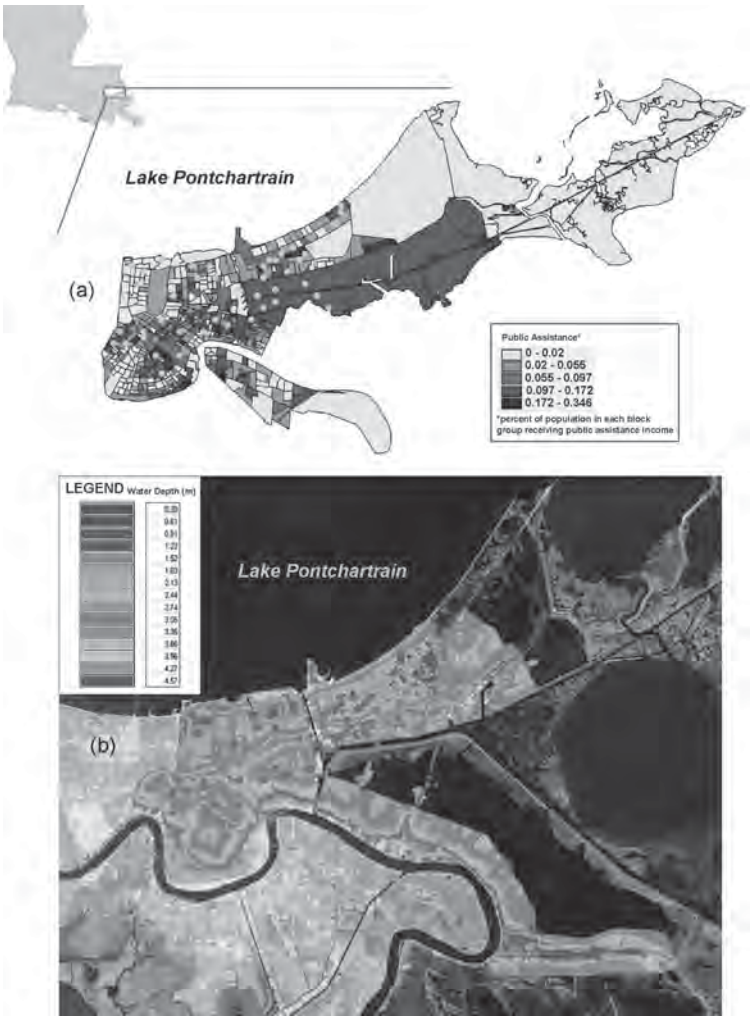

Fig. 23.1. (a) The distribution of deaths related to Hurricane Katrina for the city of New Orleans overlaid onto a choropleth map of public assistance, as defined by the percentage of the population on welfare. (b) The distribution of Katrina-related deaths overlaid on a raster grid of post-levee failure floodwater depths throughout New Orleans. *Floodwater data developed by the U.S. Federal Emergency Management Agency's Joint Field Office, September 2005*. See color plates.

process resulting in that pattern. For example, it might be found that disease "A" clusters in residences close to a water body. A specific spatial hypothesis can then be tested to determine why those spatial associations occur. For example, *elevated disease incidence is found in poor neighborhoods containing water bodies*. Or more specifically, *elevated disease incidence is found in poor neighborhoods containing water body "Y."*

The hypothesis might further state that people in poverty living close to a shared water supply (the river) are more vulnerable to waterborne diseases as compared to residences which are further away and rely on well-drawn water. This hypothesis could be tested within the GIS by comparing neighborhoods that are (i) *proximate to the river and are in poverty*, (ii) *proximate to the river but not in poverty*, and (iii) *not proximate to the river but are in poverty*.

As previously mentioned, the location of the GIS investigation also impacts the quality of available data, which in turn limits the sophistication of the analysis. In developed countries, GIS data for urban areas, in the form of street networks with encoded address ranges, building footprints, census data, and *georegistered* base maps, are often available. These data sets are less available in developing country towns and villages, and especially in more rural areas. In these environments census data are often outdated or nonexistent. Few, if any, maps are available, so either satellite imagery or aerial photography must be georegistered (spatial orientation assigned to the imagery within the GIS) in order to provide a dynamic GIS base map. Due to the lack of digital street networks in these areas, most individual disease cases or residential location data are recorded using a handheld GPS.

This is not to say that all GIS data are readily available during times of need in developed countries. The authors of this chapter were tasked to provide GIS support during the initial response of Hurricane Katrina. Working at the Emergency Operation Center (EOC), a multitude of maps were generated to help first responders understand the geography of New Orleans and its surrounding parishes. It became evident at an early stage that many important data layers were not readily available. However, most were either in existence with another local, state, or federal agency, or could be created by the GIS teams working at either Louisiana State University or the Federal Joint Field Office. Luckily, the United States, and Louisiana in particular, can be considered a GIS data rich environment. Figure 23.2(a) displays a snapshot of the "Atlas" web site home page hosted by the CADGIS computer lab at Louisiana State University (http://www.cadgis.lsu.edu). This web site is a clearing house and provides a variety of national and local GIS data sets for download. Available data include (but are not limited to): the U.S. National Wetlands Inventory, 1:24,000 Scanned Topographic Maps (DRGs), 1:100,000, and 1:250,000 Scanned Topographic Maps, Coastwide 2001 scanned images, Digital Orthophoto Quarter Quadrangles DOQQ), 1:24,000 Digital Elevation Models, and LIDAR data (Fig. 23.2b). Full descriptions of these data sets are available through the web site. The Atlas web site, and the CADGIS lab provided or generated much of the GIS data needed for the Hurricane Katrina response, while working in collaboration with the Louisiana LAGIC GIS lab, WHOCC, the LSU Hurricane Center, the LSU Coastal Studies Institute, the LSU Cartographic Information Center, the LSU Department of Geography and Anthropology, and the Louisiana Geological Survey. In addition, a new data browser and downloader were developed as a result of the disaster response to preserve perishable digital data and provide a clearing house for disaster research and planning (Fig. 23.2c). Although still in beta phase, this allows a GIS user to browse the affected region as a series of map quadrants, and access all data layers contained in a format suitable for most GIS package.

23.1.2 Why Geography Is Important

Before we further investigate the individual components of a GIS, it is useful to take a step back and return to the continuum of the spatial epidemiological investigation. In order to understand how landscape, climate, flora, and fauna fully interact with the pathogen, host, or vector, a geographic appreciation is required [20]. The location of disease cases can help develop an initial understanding of an outbreak of unknown etiology. Questions can be asked such as (i) *Where does the disease occur?* (ii) *What is the temporal gap between cases*, or (iii) *Is the spatial pattern clustered or random?* The following examples provide an introductory overview of how a GIS could theoretically be applied to different disease investigations.

23.1.2.1 Situation 1: a disease of unknown etiology

Cases of a hemorrhagic fever have been found scattered throughout Country X. The coordinates of these cases are plotted onto a country map containing various environmental variables. A remote sensing approach might investigate spatial relationships between vegetation, soil type, and disease location. A vector GIS approach might consider spatial relationships at the local level, searching for clusters or hotspots within the surveillance data – the presence of which could indicate concentrations of an environmental association, diffusion mechanisms, or even the index case. A combined approach with the two technologies would involve spatial associations with both environmental and anthropogenic data, for example, *how many cases occurred within 20 m of water bodies of a certain temperature, and houses with straw floors*. The combination of approaches is necessary because environmental data are usually extracted from remotely sensed imagery, while anthropogenic data are usually collected on the ground, the resulting GIS containing both raster and vector layers. If the hemorrhagic cases were found within an urban environment, and especially in a developed urban environment, the vector GIS allow for advanced analyses of disease diffusion and behavior. Once cases are matched to existing road files, domicile information such as building footprints, or socioeconomic characteristics could be *linked* to the addresses of the victims. Other neighborhood level data, such as the presence of an industrial plant, or proximity to a city sewer system, can also be input to expand the GIS. Hotspots can

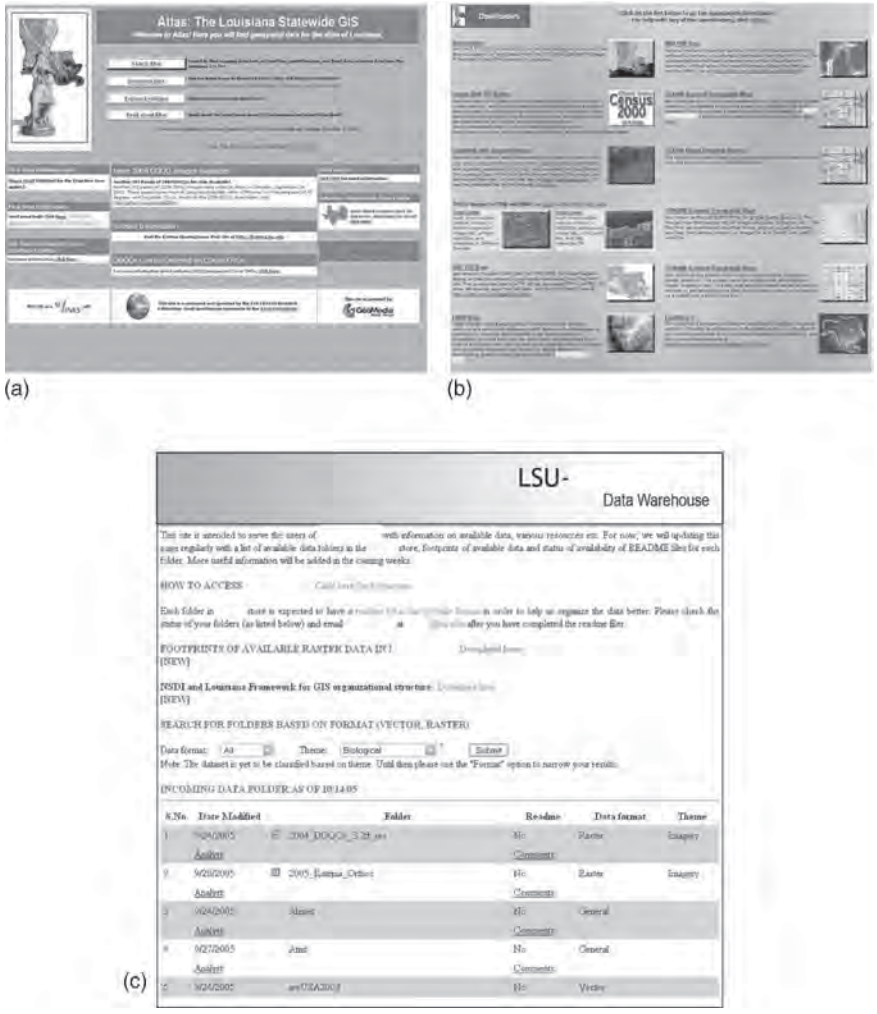

Fig. 23.2. (a) Screen capture of the Atlas web site, a GIS data clearing house managed by the CADGIS lab at Louisiana State University. (b) A screen capture of the data downloader's available through Atlas. (c) A screen capture of the data clearing house developed in response to Hurricane Katrina. Atlas is a traditional client-server database, where the user can request data from the server, but cannot write to the database (http://atlas.lsu.edu).

be identified by either finding significantly high intensities of disease cases on the map, or by identifying significantly high rates across the map. The advantage of using a rate calculation would be that the underlying population density is controlled for. This population *denominator* could either be the entire human population, or a subpopulation identified as being "at-risk." A real-world example of a rate used for spatial analyses would be the infant mortality rate, calculated as the number of infant deaths divided by all infant births within the same study area [7].

23.1.2.2 Situation 2: diseases of known etiology and partial ecology (where ecology can be defined to include anthropogenic and social characteristics) Anthrax remains a problem for livestock throughout much of the world, including both developed and developing countries. The causative agent for the disease is the bacterium *Bacillus anthracis*. While several studies discuss the environmental conditions that promote anthrax infections or *B. anthracis* spore survival in discrete study regions [15,19,61], limited quantitative work has been done on the ecology of the disease. Historical animal cases of anthrax are scattered across Kazakhstan, with surveillance data being recorded at the smallest municipal level, often village or town (Fig. 23.3a,b). In the absence of herd data, a point-in-polygon technique is used to aggregate the cases to hexagonal grid cells of varying sizes (Fig. 23.3c,d). These hexagonal cells are used to control for geographic space, while a separate gridded data set of estimated livestock densities is used to control for unknown animal populations (map layer not shown), to ensure that disease clustering techniques are not capturing whole herd population hotspots instead of disease hotspots. Once the disease

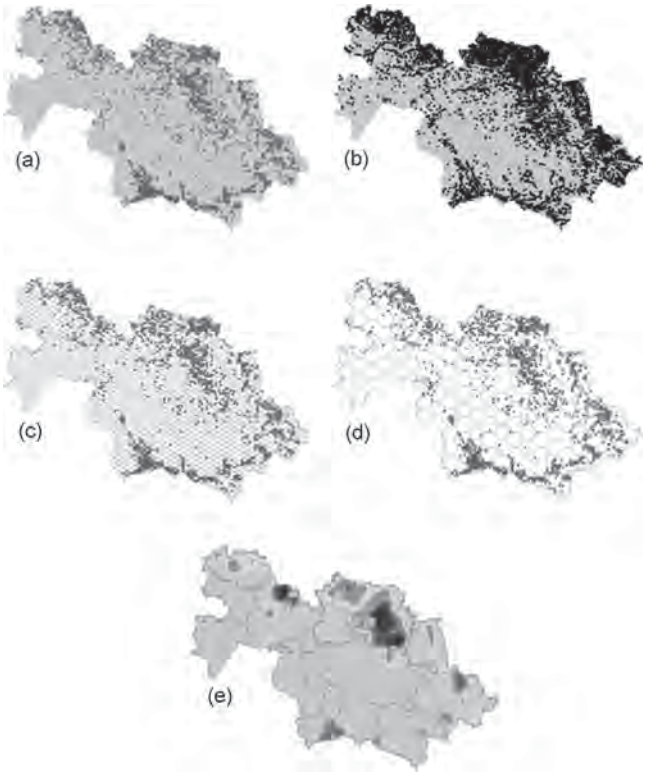

Fig. 23.3. (a) A point map representing the distribution of livestock anthrax cases for the country of Kazakhstan from 1937 to 2004. (b) The distribution of villages with fewer than 1000 individuals. (c) The distribution of livestock cases overlaid on a 25-km hexagonal grid surface. (d) The distribution of livestock cases overlaid on a 75-km hexagonal grid surface. Grid surfaces are used for aggregating data through the point-in-polygon process and useful for standardizing geographic space for analyses. (e) A kernel density surface of both livestock cases (red) and villages (blue) indicating that anthrax distributions are not completely dependent on the distribution of human population (used here as a proxy for livestock population data). Density outputs are mapped as standard deviations, shown here are the second and third standard deviations of density values. See color plates.

hotspots are identified and separated from whole herd hotspots, analyses can be performed to determine ecological relationships between *B. anthracis* and soil type, soil pH, soil moisture, disease seasonality, and herd migration practices.

In an urban example, a GIS can be used to map the spread of HIV/AIDS, a highly contagious, sexually transmitted viral disease that induces immune deficiency and secondary infection across a US city. The GIS can be used to develop an understanding of the social dynamics of the disease through space and over time in terms of how cohorts and neighborhoods evolve into a high infection status. By comparing the social information of the infected neighborhood, risk factors specific to that urban environment can be identified and mitigation strategies implemented. Figure 23.4 (insets a–c) illustrates this example with the diffusion HIV/AIDS cases for three different time periods overlaid onto a choropleth map showing neighborhoods defined by income (see section below on choropleth mapping).

23.2 THE BASIC GIS: INDIVIDUAL COMPONENTS

23.2.1 Spatial Data Input

Data input into a vector GIS investigation of infectious disease generally contain two types, spatial location (either as a single location or as a spatial aggregate) and attribute information (such as how many people live in the house, or how

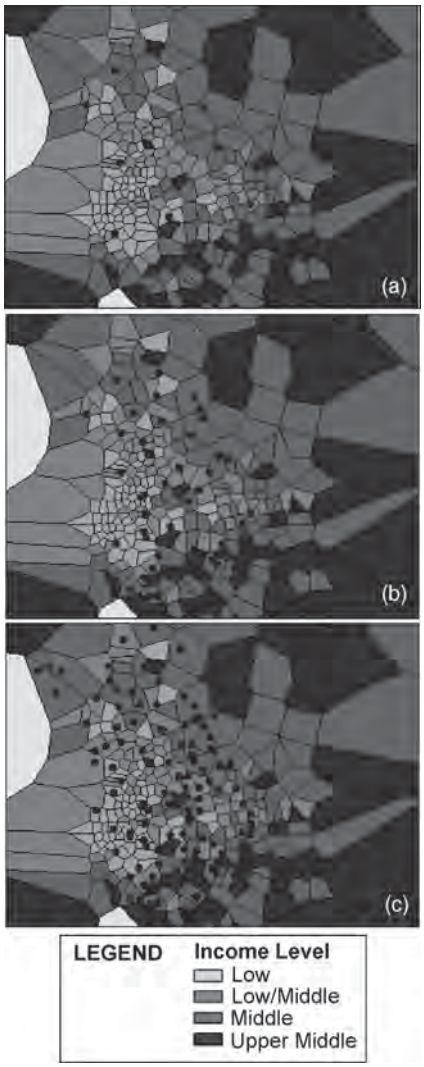

Fig. 23.4. The distribution of HIV/AIDS cases over a 10-year period. (a) The distribution of cases in year 1. (b) The distribution of cases in year 5. (c) The distribution of cases in year 10. The background vector layer is a choropleth map of income level based on US census data. The irregular polygons were developed using a technique that assigns a polygon vertex exactly halfway between any two census tract centroids. This technique is useful for masking recognizable geographic boundaries to protect victim confidentiality, while still representing the relationship between income level and HIV/AIDS distribution. See color plates.

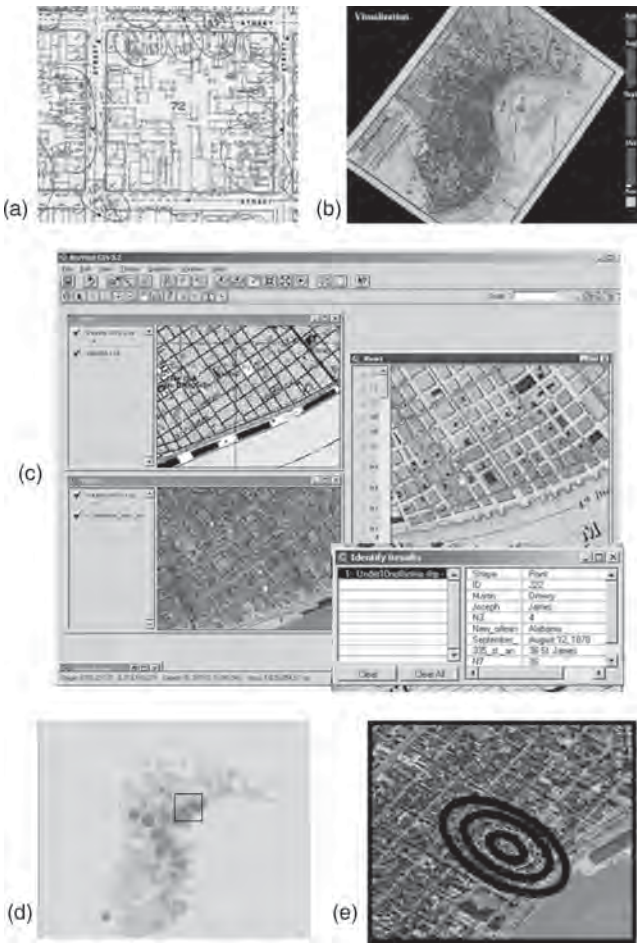

Fig. 23.5. (a) Cases from a historical role of 1878 yellow fever mortality events for the city of New Orleans, these were heads-up digitized using contemporary maps. (b) The cases were geo-registered to a map of the city and then used to develop a cartographic animation of the epidemic from beginning to end (www.whocc.lsu.edu). (c) A screen capture illustrating the dynamic link between the data table and the map window in a GIS package. Here a screen capture from ArcView 3.2 is shown with the cases mapped over a contemporary base map (ESRI, Redlands, CA). (d) A kernel density analysis of the 1878 epidemic. The inset box indicates the French Quarter hotspot. (e) Inset of (d) indicating the French Quarter hotspot. In this case, the hotspot is contoured using a technique known as Inverse Distance Weighting and then overlaid onto an aerial photo of modern day New Orleans.

many houses are found in the county). Spatial location could be stored as a recognized coordinate such as latitude/longitude, a Cartesian coordinate specific to a particular project, a residential address linked to a road network, or a heads-up digitized location (see the section below on heads-up digitizing).

Whether the data are recorded as single cases, or as spatial aggregates, other nonspatial attribute data are usually attached to the location. Examples of attribute data for a single case could be the age, race, date of onset, or date of death. For spatially aggregated data attribute fields might include total number of cases, seasonal distribution, cases by age, sex, and so on. These data, once input into the GIS, are dynamically linked to the map, meaning the user can click onto any point and see the associated data fields stored in the accompanying database. An example of this dynamic link between a point on a map and an *identification window* can be seen in Fig. 23.5(c), showing a map window and a selected case from the 1878 yellow fever epidemic of New Orleans.

23.2.2 Spatial Precision in the Data

In the previous section mention was made as to how spatial data may be available at either point or the aggregate level. This level of *spatial precision* dictates the quality of both the analysis and visualization that can be performed. In general, the greater the data precision, the more powerful the tool set that can be applied to a search for patterns and process. Returning to the John Snow cholera investigation, if cases of cholera had been recorded at a political unit level (such as census/ward/tract), no discernable pattern may have emerged because the spatial pattern (proximity to the well) occurred at a geographic scale finer than the level of aggregation. If disease cases are only available as an aggregated total, for example, for the entire city, then it is difficult if not impossible to identify what other spatial variables may have contributed to the outbreak. In another example, after Hurricane Katrina, almost the entire population of New Orleans was evacuated. This city traditionally suffers from one of the highest African American infant mortality rates in the United States. However, if only the citywide rate was considered, the neighborhood effect would be lost. It would be more useful for health workers in the refugee centers or storm shelters concerned about a *stress legacy effect* to know the characteristics of the specific neighborhoods in which pregnant mothers originally resided before the storm, rather than knowing just about New Orleans.

A similar problem occurs when zoonotic diseases are recorded as aggregates. Rabies is a zoonotic disease belonging to the viral family *Rhabdoviridae* found in many wildlife species that interact with humans in urban and semi-urban environments throughout much of the world. If rabies surveillance is recorded by county, it is hard to associate diffusion to environmental features, such as river valleys or forest margins, simply because actual case locations are unknown [6]. This problem magnifies as the spatial aggregation becomes larger. In South America, animals submitted for foot and mouth disease testing are initially located on a relatively fine spatial grid. However, when positive cases of the disease are determined by laboratory analysis, they are reported back by municipal unit, which covers far greater geographic areas. The problem of aggregation occurs for both surveillance and positive data sets, but the potential to look for spatial association has decreased when considering just the positive cases.

23.2.2.1 Modifiable area unit problem: redefining a disease quotient with GIS If data are only available in a spatially aggregated form, care must be taken that analytical results are not merely an artifact of the available level of spatial aggregation. This *modifiable area unit problem* can occur due to an aggregation effect (a problem of scale), or due to the shape of those aggregations (a problem of *zones*) [9]. To illustrate this problem, consider the approach to determine disease risk often employed in the former Soviet Union, a *disease quotient*, which is expressed as follows:

$$\text{Risk} = (n/t)/(N/T)$$

where "n" = cases in a town, "t" = time periods when cases were found, "N" = total number of cases, and "T" = total time periods when cases were found. Traditionally the measure for "total" is the political unit containing the town, such as a rayon or oblast. The final expression gives a risk proportion for that town based on the total risk for the surrounding area. Unfortunately, this risk proportion is dependent on the size of the political unit. The risk proportion will never change for the town, though it might lessen, or even increase, as the size of the geographic area changes. A town may be a *cold spot* (an areal unit that lacks a significant number of disease cases surrounded by areal units with significant numbers of cases) in a cluster of neighboring towns with high amounts of disease. However, the disease load for the cold town may be high when compared to all towns in the rayon. This is a problem of geographic scale. In the absence of GIS technology, there is an obvious reason to choose political units – the collation and calculation of data in a traditional database need a consistent and recognized unit of aggregation. With a GIS, however, and presuming the initial surveillance data exist as point locations, the geography of the aggregation can be varied in both scale and geometry. It is important to investigate multiple scales and zones in order to identify consistency in the spatial pattern. In other words, modifying scale is useful in determining whether or not towns have elevated risk of disease infection irrespective of what geography of aggregation is chosen.

23.2.3 Data Entry into the GIS: Geocoding, Entering Coordinates, Heads-up Digitizing

23.2.3.1 Geocoding Presuming spatially precise data exist, there are three common methods in which these point data are entered into a GIS. First, the address of the disease case can be *geocoded* or address matched as a point to an existing street file (line network) of the city. This technique is reliant on the underlying street network data and, therefore, usually only available in urban areas. For many cities, road files are available with address ranges encoded onto the line segments. The GIS proportionally places the address along the correct road segment according to these ranges. For example, if an address is 30 Washington Ave, and the road segment ranges from 0 to 100, the address (a point) will be placed 0.3 line length units from the 0 location. A *batch process* can be used to locate thousands of addresses simultaneously, though errors in data entry, road name changes, new roads, alternative names, and misspelling can all affect the percentage of correctly matched addresses. A second interactive rematch, with the aid of a city map and Internet search engines, can reduce the total number of misidentified addresses further. A 90% success would be considered acceptable in most circumstances. The final 10% might include addresses that are impossible to match, such as post office boxes [28,42,50].

During Hurricane Katrina, GIS personnel working at the EOC were often requested to perform tasks requiring geocoding. Two typical tasks included (i) locating hospitals and health clinics so that U.S. Public Health Service field teams could identify local medical needs, and (ii) mapping emergency 911 calls and providing geographic coordinates of addresses so that first response teams could locate callers via GPS navigation. This was especially important in the immediate areas surrounding New Orleans that were inundated with water from the levee failures and rescues were performed by boat (see Fig. 23.Ib for areas in water too deep to navigate by vehicle and street signs).

23.2.3.2 Entering coordinates A second way to enter point data is by collecting traditional geographic *coordinates*. This generally is done in one of two ways, either by locating the position on a field map, or more commonly now, recording the location using handheld GPS receivers. The advent of two new technologies have dramatically improved the way spatial data can be recorded, uploaded, and analyzed in the field. The first of these is the 12-channel personal GPS unit. As long as the field researcher does not need data recorded with greater than 10-m precision, many handheld units costing less than US $100.00 are readily available. A field researcher, such as the one shown in Figure 23.6(b), collecting Chagas disease data in Mexico, can record both spatial location and attribute information (for each house type, or whether a specimen jar has been left at a residence) on a Personal Data Assistant (PDA) handheld computer running a field GIS application. Figure 23.8 displays hotspots of West Nile Virus (presented as state-managed mosquito abatement zones) for East Baton Rouge Parish in Louisiana, United States. The points used to determine these hotspots are also shown; the human cases were address matched, and the bird locations were recorded using GPS receivers.

In the immediate aftermath of Hurricane Katrina, three researchers from the WHOCC joined disaster assessment teams sent into the field by the U.S. National Red Cross. Following the storm, New Orleans was an exclusion zone due to the floodwaters, street violence, and ongoing search and rescue operations. During that time several Red Cross teams focused on the other parishes in the affected area. The usual disaster assessment tool utilized by Red Cross field teams includes a visual identification of each house type (single family home, mobile home, apartment), and a standardized classification of the degree of damage sustained. These data are used to distribute Red Cross emergency funds to those affected by the disaster, and by the U.S. Federal Emergency Management Agency (FEMA) to characterize overall damage. Before Hurricane Katrina, these data would remain largely as paper files; however, a team from LSU entered all data into a GIS allowing for damage mapping and querying by attribute field. In addition, the WHOCC field team also captured GPS-linked digital images in parishes that were particularly rural. Fig. 23.7(a) displays points captured on one assessment trip into Washington Parish, along with sample images from the locations (Fig. 23.7b–d).

Internet-based GIS and data entry: An emerging technological advance in spatial data collection is the availability of the Internet as a means of accessing remote data storage/analysis/visualization applications. This connectivity allows the field researcher to upload data from a field station, or via a cellular connection. The WHOCC has been developing web-based GIS/database applications for field researchers who either do not have access to GIS software, or do not have the required GIS expertise. This online GIS is built on *Active Server Pages* technology and allows the user to enter data into a database housed on a server. Those data are then dynamically linked to an Internet mapping application that reads from the database and displays data events as points over study area imagery as they are entered. The online database portal contains several previously designed data forms specific to the disease investigation. The field researcher uploads the information he/she has collected along with GPS-derived coordinates or locations read from a paper map, into these data forms from the field station. These data, including all the attribute information linked to each coordinate, can then be displayed and queried using the tools built into the web-based GIS. The functionality of this web GIS is limited in comparison to desktop applications, but still offers non-GIS personnel the opportunity to display the data and a means of performing simple queries. For example, it is possible to identify the location of all houses with bugs testing positive for *Trypanosoma cruzi*, the causative agent of Chagas disease. Additionally, the user can export the data from the web-GIS to non-GIS spreadsheets for aspatial analyses or report generation. The benefit of this system is twofold: (i) all data are uploaded and stored in a secure environment, and not solely on portable data storage devices or laptops that are subject to rigorous field conditions, and (ii) these data can be analyzed by GIS personnel back at the research laboratory with results being immediately fed back to the field. For example, a typical dialogue between the field researcher and the lab personnel might be a modification to the spatial sampling strategy to keep the distribution of houses with insect collection jars in line with published findings of sample strategies [45]. All of the above stages are illustrated in Figure 23.6 (insets a–f), which displays an ongoing research project in the Los Tuxtlas region of Veracruz, Mexico. In this project, insects were collected via voluntary household collections, coordinated through an ethnography graduate student, and sent from the field to Loyola University in New Orleans for *T. cruzi* identification using PCR analysis. As soon as these results were available, they were sent electronically to WHOCC personnel who, within a 24-h period, presented the findings at a professional meeting and at the same time uploaded them to the web-GIS. Both the audience at the meeting and the researcher in Mexico were shown which houses were positive for the disease at approximately the same time. This type of data collection mechanism should dramatically improve the quality of spatial data, provided that a host university or GIS lab can provide the required expertise and web-based data entry support. As long as the field

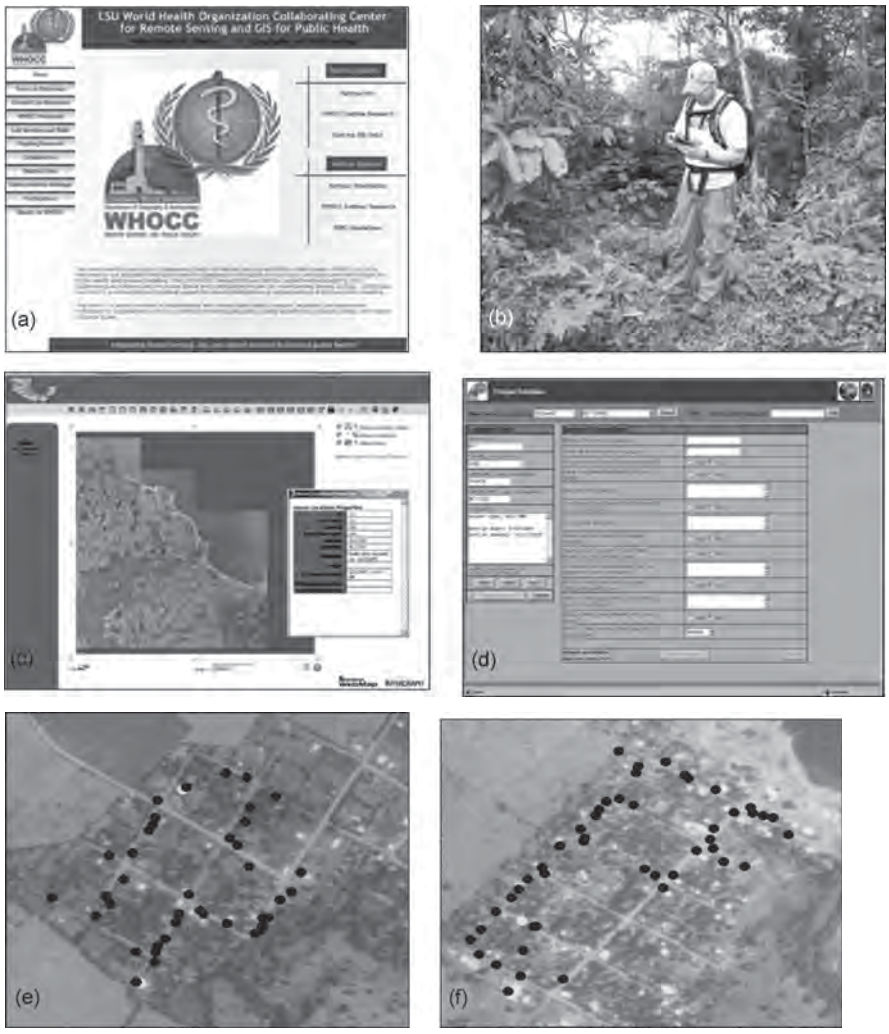

Fig. 23.6. (a) A screen capture of the LSU WHOCC web site used as a portal to the Chagas online data entry system and the Chagas map web-GIS. (b) WHOCC researcher recording Chagas-specific field data with a handheld PDA running integrated GPS/GIS software. (c) Screen capture of the Chagas web-GIS. The table in the right-hand corner of the inset corresponds to the data entry screen of inset (d). The points on the map indicate the distribution of sample bottles for disease vectoring insects. (d) A view of the online data entry forms. (e,f) Close-up images of two villages where samples were collected. The red dots indicate houses where bottles were distributed for voluntary insect collections, and the yellow dots indicate houses where insects tested positive for *T. cruzi*. See color plates.

personnel have network connectivity through an Internet Service Provider or a cellular connection, this technology can make field data spatial analysis "near" real-time.

23.2.3.3 Heads-up digitizing The third method of point data entry is known as *heads-up* digitizing – named for the manner in which the user interacts with a digital map on screen with eyes-looking-up. For this approach a base layer, such as an aerial photograph of a city, or in the John Snow example, a map from 1853, is used to visually identify where locations should be captured as a new GIS data layer. This is achieved by simply clicking on top of the desired location with the computer mouse. If the base layer has been georegistered, which means it contains known spatial coordinates and acts as a functional GIS map, these "clicked" points are saved as coordinates that can be transferred to other GIS layers, or even to other GIS applications. Points, lines (such as a drainage ditch), and polygons (the footprint of a house) can all be developed with this method. Figure 23.5 displays this process by showing the transition from a historical mortality role of the 1878 yellow fever epidemic of New Orleans (Fig. 23.5a), to the output of a hotspot analysis of this point data identifying the French Quarter.

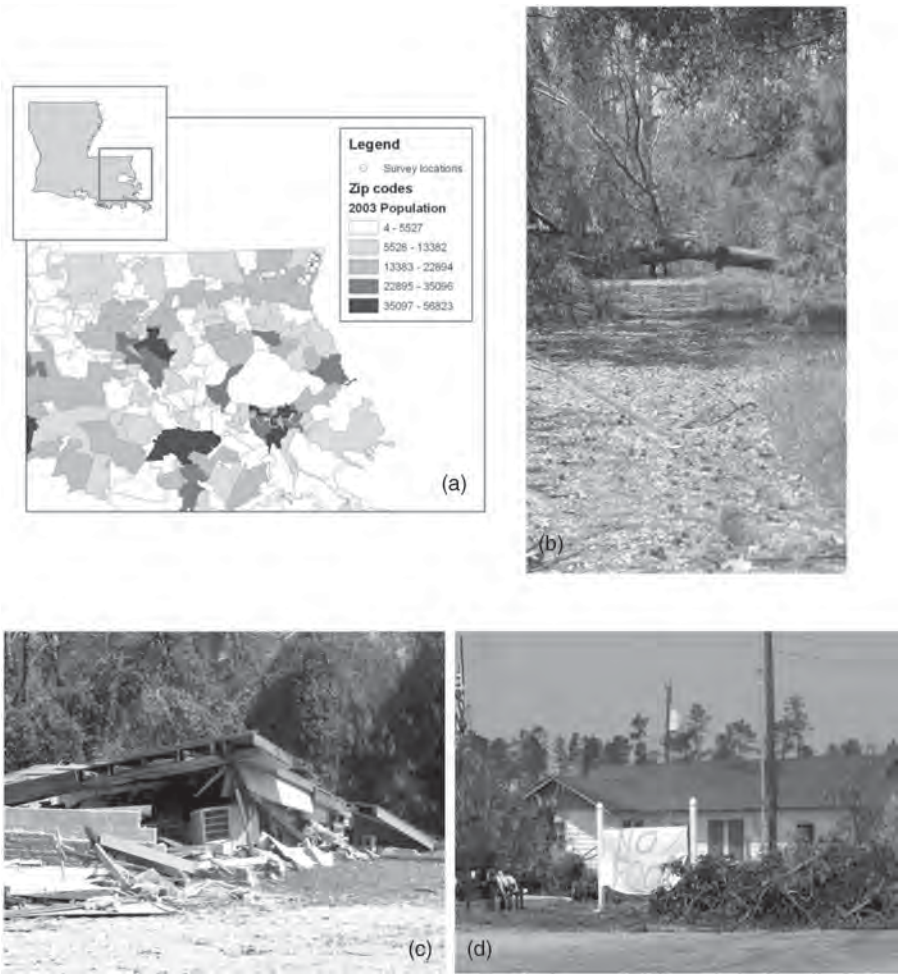

Fig. 23.7. (a) Data points collected in affected rural areas as part of the WHOCC effort to aid Red Cross in the aftermath of Hurricane Katrina. Rural is indicated with a choropleth map of zip code boundaries based on US census data. (b) An example of the rural roads and downed trees within the survey area. (c) An example of a destroyed residence within the survey area, giving some indication of the power of the storm. (d) An example of residents communicating a post-storm need in a community with no power and no phone communication.

During Hurricane Katrina heads-up digitizing was a frequently used means of creating GIS data that were not previously available. One example was the development of hostile area polygons for use by joint police task forces to identify areas of street violence and aid in the development of a security strategy for regaining peace and lawful control.

Joining data tables with GIS layers: Spatially aggregated data can be entered into the GIS by joining tables based on common fields. For example, a researcher may want to understand the spread of West Nile Virus – a mosquito-borne disease. The first step would be to open a GIS layer of US zip codes. A table, created in a spreadsheet or database package, contains the number of cases by zip code, by month, and by year. When this data set is imported into the GIS, it can be *joined* to the zip code map by a common zip code identifier. The user can now make maps by month, or by year, to show how cases of West Nile Virus have diffused across the country.

23.3 DATA MANIPULATION

The major role of a GIS in an epidemiological investigation is as a tool to manipulate data both spatially and aspatially. One very common GIS manipulation is the aggregation of data to alternative spatial units. For example, if data exist as points, such as the location of houses with insects positive for *T. cruzi* cases, these can be displayed by aggregating into any traditional geographic unit (e.g., the town, the province) or as new geographies, such as buffer zones extending out from the forest margin, hexagons representing equal areas of space (Fig. 23.3c,d), or asymmetrical polygons developed mathematically (Fig. 23.4a–c). There are many reasons why points should be spatially aggregated. Many thematic maps require aggregates rather than point locations (e.g., the choropleth map of public assistance in Fig. 23.1a). Similarly, many forms of spatial analysis require aggregate units. Figure 23.3 (insets

c and d) display two hexagonal surfaces onto which the point locations of anthrax occurrence for Kazakhstan have been aggregated (using the point-in-polygon technique previously described). This aggregation standardizes space, so rates for equal land area units can be input into a spatial cluster analysis. The GIS makes this spatial transformation from point data to any aggregation a relatively easy transformation. The ability to change aggregations is also a necessity if multiple spatial data sets are to be used in the analysis (such as census data being combined with disease presence). In this case a common spatial unit of analysis is needed. Being able to change geographic aggregates also reduces the potential for spurious analysis results which are merely an artifact of the geographic scale under investigation (the previously mentioned disease quotient being one obvious example). Finally, aggregating data helps preserve patient confidentiality by spreading cases of disease over large denominator populations.

Flexibility in spatially aggregating events is dependent on the original spatial precision of the collected data. Diseases are frequently continuous over space, with natural or environmental barriers more likely providing edges to an epidemic than a political boundary. Therefore, being able to aggregate point data to any environmental area rather than to a traditional spatial–political unit is advantageous. Several references are provided on aggregation effects in health analyses [33,34,56,57].

23.3.1 Querying Data

The common way to reduce a data set based on aspatial attributes is through a query. Much in the same way a traditional database works, a GIS query is built as a Boolean expression, which is a means of questioning the database using an algebraic language, for example "Age $<$ 10." This query would search the attribute column "age" for any mortality occurring to individuals under the age of 10. Only those mortalities that meet this query are selected out, and if necessary saved as a new GIS layer. A map of this new GIS layer would show the spatial distribution of children less than 10 years of age in the epidemic. Statistical and spatial analyses can also be performed on the subset, such as identifying if children were more susceptible at the beginning, middle, or end of the epidemic. The added advantage of the GIS is that an additional spatial query would focus this question into a specific geographic area. For example, it might be believed that children living close to the docks experienced more continuous disease exposure and, therefore, had a higher level of immunity. The initial query would be modified to include *only those children living within 0.25 miles of the docks*. From these simple manipulations three comparisons can be made – children compared to all mortalities in the epidemic, children in the buffer area compared to all mortalities, and children in the buffer area compared to all children in the epidemic. Aspatial attributes attached to geographic locations can also be manipulated to change the data under investigation through a GIS query.

23.3.2 Spatial R_0: Spatial Querying

Typical GIS queries might involve the reduction of the population under investigation by a particular cohort, or date of death, or a travel pattern. One way to illustrate how useful these queries can be is by using the GIS created for the 1878 yellow fever epidemic of New Orleans (Fig. 23.5). A mortality role published immediately after this epidemic contained the names, nativity, age, date of death, and residence of those who died in the epidemic (Fig. 23.5a). The spatial distribution of the epidemic was heads-up digitized as a GIS layer using contemporary maps. By using GIS queries (Fig. 23.5c) a spatial basic reproduction number (R_0) was calculated for the mosquito – vector of yellow fever. The R_0 determines how many deaths can be linked to a single introduction of the disease into a neighborhood. The first query identified the first mortality in each neighborhood. Only those "index cases" that did not have an earlier death occurring within 50 m of the mortality residence (approximating the flying range of the mosquito) were kept. This reduced data set now included those mortalities that were probably the first disease introduction into that neighborhood. A further query reduced the number of these index cases by only selecting those buffers where a second mortality occurred at least 13 days later. This time frame allowed for the extrinsic incubation period of the mosquito to be considered. In this way the second mortality can be linked to the index case, meaning a mosquito feeding on the first victim then caused subsequent deaths in the neighborhood.

23.3.3 Caution with Aggregation and Disaggregation

Other types of manipulations could include the combination of data sets to form rate surfaces, or indices based on multiple variables (a deprivation index being one of the more common examples) [25–27]. However, researchers must also be careful of causing errors by attempting to disaggregate aggregate data. For example, one common manipulation would be to assign the social variables of a neighborhood (extracted from the census) to a disease case living in this neighborhood. The reason for this manipulation would be to analyze whether disease could be associated with different neighborhood characteristics, a commonly used approach in public health multilevel modeling [11,12,29,32,41]. For example, if a census unit states that the median house value for a given aggregate is $50,000, a problem may arise when a case of tuberculosis is assigned this value (as a proxy for household wealth) to show how poverty and a failure to complete antibiotic regimes lead to resistant strains of the disease. The house in which the individual lives may be worth $10,000 or $100,000. Without other information this is a best guess. It should be noted, however, that a time-consuming evaluation of contemporary maps or aerial photography can help identify further spatial detail into the aggregate geometry by matching the address of the victim to the type of house structure in which he/she resides.

23.4 SPATIAL ANALYSIS

One of the most powerful advantages of using a GIS in an epidemiological investigation is the spatial analytical capability. If we return to the initial John Snow example, an exploratory point map revealed a common infection source (the Broad Street pump). A GIS would now allow for a test of whether or not the pattern was statistically significant. This can be expanded beyond the identification of patterns to the determination of other spatial associations. This is the first step toward developing a spatial hypothesis that can be transferred and tested in other locations and for other time periods.

Spatial analyses and the general use of statistics in a spatial environment can range from basic to advanced. GIS queries can be used to create reduced datasets. Descriptive statistics (sum, range, mean, minimum, maximum, variance, and standard deviation) for these subsets can be used to compare similar geographic areas, or a comparison of the subset against the entire population for differences in disease distribution. For example, one could evaluate *if insects collected in a Mexican village are more likely to be infected with T. cruzi if the house from where they are collected is close to the forest edge.* By comparing insects testing positive at different distance bands (these distance bands being extracted through a GIS query), a univariate relationship between distance from forest margin and parasite prevalence can be determined through statistical testing. A recent approach to the GIS analysis of Chagas data involved the identification of those areas within the village that appeared to facilitate re-infestation after insect control measures were implemented [24]. Cluster techniques can be used to identify not only where a hotspot is located but also how large of a geographic area the hotspot covers. Therefore, a woodpile can be identified as the center of the cluster, and that houses x meters away become infested over y time periods. Similar techniques have also been used to determine the local spatial variation in mosquito-vectored diseases [21,46,47,59].

A similar approach could be used to spatially analyze anthropogenic data, such as the perception of Chagas risk in different communities. One might test *whether there is a decreasing fear of the disease the further the hut lies from the forest edge*. Data from a simple questionnaire, possibly uploaded through the previously described web-GIS, could be analyzed using one of a series of traditional statistical techniques (e.g., cross-tabulation, odds ratios, *t*-tests). These data are again analyzed by different spatial manipulations; *compare Village A to Village B*, or *compare houses within 100 m of the forest edge to those beyond 100 m*.

23.4.1 Kernel Density Analysis

Of course, more sophisticated models designed specifically for spatial data are also available in the GIS environment. The figures in this chapter display examples of some commonly applied techniques. Both Fig. 23.3(e) and Figure 23.6(d) display kernel density analysis used to identify hotspots of disease; anthrax in Kazakhstan (1937–2004), and yellow fever in New Orleans (1878), respectively. Kernel density analysis is a deterministic geographic interpolation technique for calculating weighted densities of events over a gridded surface within a kernel, or spatial bin [17]. Although different density functions exist for the calculation within the kernel, the most common function applied approximates the standard power based distance decay curve. Whereby, events occurring closer to the centroid exert greater influence on the density calculation than those closer to the kernel edge.

This interpolation technique creates a smoothed density surface, either using a single disease case or weighted by an additional attribute, such as the number of animals that died at that location. The resulting surface is a grid containing a density value in each cell. By classifying these cells into standard deviations, hotspots exceeding two standard deviations can be extracted. In Figure 23.3(e) an anthrax hotspot is seen in the southern section of Kazakhstan (red). In Figure 23.6(d) a yellow fever hotspot is located in the New Orleans French Quarter (also identified on the aerial photograph Fig. 23.6e). Figure 23.3 also displays a second kernel density surface for villages in Kazakhstan (blue). As the historical anthrax data set contains no denominator values of herd size, a comparison of the disease density surface against human settlement densities can be a useful proxy for identifying areas where anthrax may be truly elevated and not elevated merely because of increased human and animal population or greater reporting. Though useful for indicating areas of interest for further analysis, deterministic methods such as kernel density should not be used exclusively. When data sets are appropriate, probabilistic models should be employed to test specific hypotheses.

23.4.2 Measures of Spatial Autocorrelation and Spatial Forms of Regression

An alternative analytical approach is seen in Figure 23.8. Here a *local indicator of spatial autocorrelation* (LISA) was used to identify hotspots in aggregated surfaces. In this example, human cases of West Nile Virus were aggregated to mosquito abatement service areas. Those service areas with statistically significant elevated disease, as compared to disease cases in the neighboring areas, are shown in red. Similarly, measures of local spatial autocorrelation have also been applied to the 1878 yellow fever data to show not only which parts of the city had elevated disease levels, but at which spatial scales these hotspots are at their maximum. Results of a Gi^{*}(d) analysis [21] indicate that 10 m, or single residences, were centers of maximum infection within this epidemic (results not shown here). This finding matches the limited flying range and general activity of the *Aedes aegypti* mosquito.

Many other space and space – time approaches have been applied to health data [36,49]. For many of these techniques, statistical significance is identified by comparing actual clusters to a distribution built from multiple Monte Carlo simulations. This allows the user to identify how frequently the

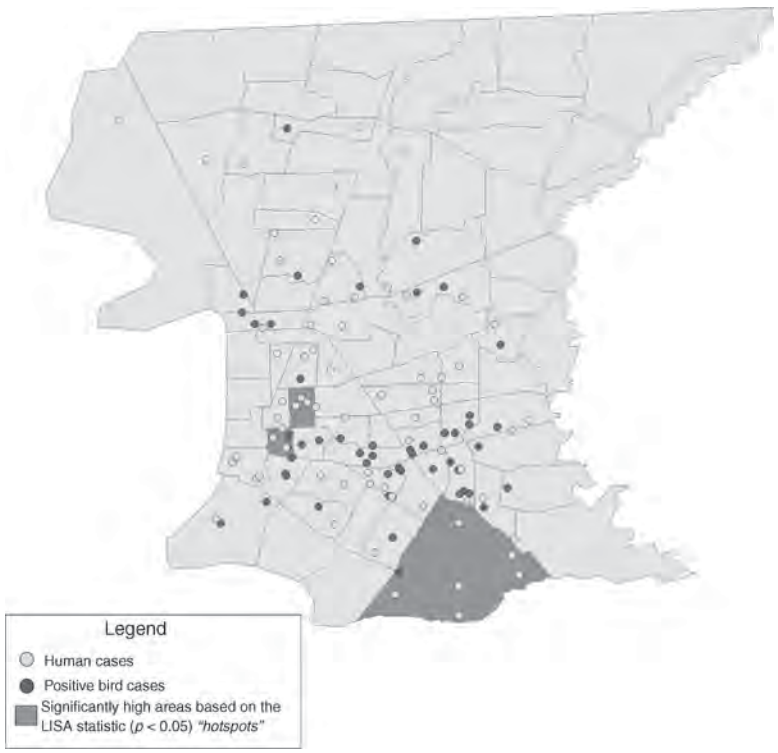

Fig. 23.8. Output from a *local indicator of spatial autocorrelation (LISA)* statistical analysis for West Nile Virus in East Baton Rouge Parish based on mosquito abatement districts. The analysis was calculated on the polygon data set once the point-in-polygon method was used to summate the number of bird and human cases in each polygon. This output represents the *LISA* statistic for bird cases. Human cases were plotted using address matching and bird cases were plotted using GPS coordinates. *Data were provided by the East Baton Rouge Mosquito Abatement and Rodent Control office.*

geographic cluster appears by chance alone [53]. In other words, given the same general conditions surrounding the disease occurrence, *how often would you expect a woodpile to have an elevated number of insects*, or *how often would you expect this city block to have x number of infant deaths*.

Spatial variations of traditional regression models are also available either within a GIS, or in spatial analysis software packages that link to a GIS. These models are used to look for local variation between dependent and independent variables, rather than assuming that a global relationship fits all localities in the same way. For example, it may be found that poverty explains HIV infection, but that this relationship diminishes in the presence of a third variable, that of an active church. It might have been possible to see this relationship in a traditional regression analysis by mapping the model residuals. However, new spatial regression models, such as geographically weighted regression, consider these local variations directly [16,17].

23.4.3 Spatial Analysis Software

Software to perform these analyses comes from four general sources, as stand-alone packages from private vendors, as software packages available through academic institutions, as Internet downloads as spatial analysis extensions to current GIS packages, and finally as freeware GIS packages often designed for the purpose of a particular GIS analysis function (DesktopGarp- http://www.lifemapper.org/desktopgarp/ is a freeware ecological niche modeling package, and DIVA-GIS- www.diva-gis.org/ is a full functioning GIS with an ecological niche modeling suite built-in).

Three widely used packages include: (i) CrimeStat® which allows for the construction of space and space time clusters in a point dataset [38]; (ii) GeoDa™ (http://www.csiss.org/clearinghouse/GeoDa/) which can be used for exploratory spatial analysis, including identifying patterns within aggregated surfaces in a dynamically linked window environment, and the LISA analysis illustrated in Figure 23.8; and (iii) Geographically Weighted Regression (GWR - http://ncg.nuim.ie/ncg/GWR/software.htm) which allows for local regressions to be fitted (the regression parameters varying across space). All three of these packages are either free or available at a minimal cost. In addition, SaTScan™ (also available for free download – http://www.satscan.org/), and ClusterSeer® (http://www.terraseer.com/) are also useful applications that contain both space and space–time clustering techniques.

Many of these analysis packages contain dynamic windows that allow the user to manipulate, analyze, and visualize data on-the-fly. For example, the problem of outliers (including spatial outliers) has long been problematic in statistical analysis. A dynamic window environment allows for the user to remove these from the analysis, with the immediate effect to both map and analysis being displayed.

In summary, GIS allows for a variety of different spatial analytical approaches ranging from simple overlay, summary statistics of queries, to identifying spatial and spatio-temporal clusters in both point and aggregate surfaces. In addition, new spatial regression models now capture local variation in spatial association. A single section of this chapter is a limited forum to cover the breadth of GIS and spatial analyses, and the reader is directed to a series of references that cover the GIS/Spatial Analysis Interface [2,17,40,58].

23.5 SPATIAL VISUALIZATION

23.5.1 Map Production

The most frequent use of a GIS is for map production. GIS data are spatial in nature, and therefore should be mapped. Although spatial analysis can help detect patterns in spatial data, the power to visualize spatial relationships should not be understated. The commonly cited example (though it seems somewhat erroneous) is that John Snow identified the origin of the cholera outbreak by viewing a mapped pattern. Maps can be used to summarize and display large amounts of disease data, such as the anthrax distributions seen in Figure 23.3, show temporal disease diffusion as with HIV/AIDS in Figure 23.4 and yellow fever in Figure 23.5, and identify where in the village infected bugs have been collected (Fig. 23.6, insets d and e).

GIS maps vary for several reasons – the type and quality of the available data, the skill of the cartographer, the available technology, the purpose of the investigation, and the map audience. With this last point it should be remembered that a GIS is not only an investigation tool but also a means of communication (which might be the real reason behind John Snow's map). For example, if a spatial pattern is found in the neighborhood suggesting an elevated likelihood of disease close to a particular wooded area, a map can be created to display that risk [35,55,60]. Risk maps can be constructed to guide health workers in outreach, to inform politicians to bring about legislative change, or to educate the public. With each audience the map must be modified. Different terminologies must be used, different points of spatial reference presented, and most importantly, the confidentiality rights of potential victims must be protected by changing the data displayed [37].

23.5.2 Protecting Confidentiality While Preserving Spatial Relationships

This chapter has already mentioned how aggregating point data *masks* disease cases by the underlying population. By displaying disease cases on an aerial photograph, or even a road network, it might be possible for an audience to identify where the victim lives. Figure 23.4 displays a simple map transformation that helps mask the underlying geography. In this case the center of each census tract was used to create new polygons, the boundary of each being exactly half the distance between each census tract center. This simple geographic manipulation allows for disease data to be displayed while removing instantly recognizable geographic features [7]. It is therefore hard to identify exactly where the HIV residences fall (for the purposes of this chapter all human disease or death cases have been randomly redistributed). Unfortunately, we are left with a situation whereby the purpose of the map may be to warn the public, and yet how can we achieve this if no recognizable spatial features are included? In one anecdotal example from the United States, a relatively small town was not informed about West Nile virus mortalities because the limited population size jeopardized the confidentiality of the deceased. Mosquito-spraying activity in the United States is based on telephone-based complaints. If the public is not aware of the threat of the virus, they are less likely to phone in mosquito complaints for strictly on "nuisance" value alone. It is necessary in these situations that the office of public health, the mosquito abatement, and local political leaders are in constant dialogue, working to mitigate such confidentiality situations in an effort to provide maximum protection.

23.5.3 Choropleth Maps

One useful map display is a choropleth or graduated color map – a standard cartographic display option in any GIS. Both qualitative and quantitative data can be displayed as choropleth maps (examples are shown in Fig. 23.1a, 23.4a–c, 23.7a, and 23.9a–f). A choropleth map is not the only type of thematic display available in a GIS. Diseases are often mapped as dot maps (where each disease case is represented by a point as in Fig. 23.3a,b for anthrax), and isoline surfaces (where data are interpolated, so contour lines display areas of similar value). The choropleth map, however, is a frequently used thematic display for public health data.

The typical choropleth map displays intensities of a variable for each polygon on a base map. In Figure 23.3 (insets c and d), a choropleth map could have been constructed for the hexagonal surface. In Figure 23.4, the choropleth surfaces have been created from polygons derived from the centroids of census tracts. In Figure 23.7(a), population is represented by zip code boundaries to indicate the survey took place in a rural area. In Figure 23.9 (insets a – f) counties are the base units. Other traditional polygons could include census units, health regions, or countries. The disease data within these units are classified (the way data are broken into different categories) according to one of several techniques. Data can be classified so as an equal number of polygons fall into each class (if there are 100 counties, and five classes, 20 counties fall into each color category). Data can be classified according to equal steps in the range of the data (if the lowest number of cases is 1, and the highest is 20, and five classes are required, those counties with 1–4 cases are colored white, those counties with 5–8 are colored light gray, and so on). Other classifications include finding the greatest natural breaks in the range, or classifying each polygon by standard deviations (how far each polygon value is away from the mean of the distribution).

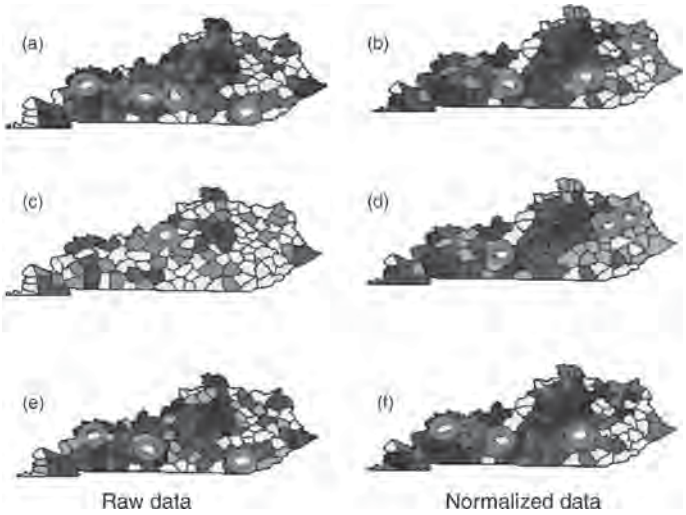

Fig. 23.9. The distribution of rabies surveillance data at the county level for the state of Kentucky using choropleth mapping comparing differences in classification techniques, the number of classes, and raw versus normalized data. (a) Equal area with four classes; (b) equal area with five classes; (c) natural breaks with five classes; (d) natural breaks with six classes; (e) quantile with six classes; (f) quantile with four classes. Red circles indicate counties with apparently no disease present. See color plates.

The type of classification chosen, along with the number of classes chosen, can dramatically affect the map pattern. Figure 23.9 displays six maps (insets a–f) created for the same rabies surveillance data set from Kentucky. Red circles display those counties where disease is apparently not present. The three classification types include an equal number of counties in each color (equal area), where the largest breaks occur in the data range (natural breaks), and an equal step in the data range (quantiles). The number of classes chosen is also varied. The data are also mapped by either the raw total number of positive cases for the county or the incidence of rabies (total number of positives divided by total number of animals tested). Similar to the warnings about different spatial aggregations leading to different analytical results, different mapping techniques lead to different map outputs and apparent spatial patterns. Therefore, it is always wise to map data in multiple ways to ensure the pattern is real and not an artifact of the map choice.

23.5.4 The Importance of Basic Cartographic Rules

This discussion leads to a final note of concern. Producing maps in a GIS has become relatively easy, with both technology and software now being affordable or even free. This ease of access is dangerous if the GIS user does not invest the time in learning some basic cartographic principles. Raw data should rarely be used to map areas of high disease presence, as it is necessary to include a measure of the underlying population, to ensure that disease presence is not just a measure of population presence. The differences in outputs between raw and normalized data are illustrated clearly in Figure 23.9. Quantitative data should be mapped using a consistent color ramp (light to dark colors, white to black being one of the best). Multiple maps should only be compared if the same classification breaks are used, or by standard deviations if the data distribution is normal (to show an increase in disease such as in Fig. 23.4). The number of data classes should generally not exceed seven, otherwise color variations are hard to detect. These are some of the standard rules of map making. The reader is pointed to a series of cartographic texts [3,10,22,44,48, 51,54].

23.5.5 Cartographic Animation

Finally, while a static map series can be used to display the temporal progression of a disease, maps created in a GIS can be output to a number of animation software applications that allow the user to view the spatial and temporal progression of the infection dynamically [8]. Innovative software, such as Flash (Macromedia, San Francisco, California, USA), allow the user to organize a series of map outputs in a "film strip" style timeline and then animate the appearance of those maps, one on the other, sequentially. For example, the user could set the timeline to introduce a new map every 1 s, or 1.5 s. Figure 23.5(b) contains a frame from the cartographic animation of the 1878 yellow fever epidemic. This type of visualization allows the user to see diffusion corridors, and

barriers, that both static maps and space–time analyses may potentially miss. The full cartographic animation of this 1878 epidemic can be viewed at the LSU WHOC web site (http://www.whocc.lsu.edu).

New analytical techniques are also being developed with this animation technology. The Space-Time Intelligence System (STIS; Terraseer Inc., www.terraseer.com) is a new GIS that performs spatial and spatio-temporal analyses, and can be used to develop cartographic animations within the GIS. STIS allows the user to link maps, similar to the discussion on linking earlier, but also allows the user to interact with a timeline tool, where by sliding a cursor over the timeline dynamically shifts the map view to that time period. STIS also allows the user to modify spatial statistics such as the LISA (see section above) in order to compare a spatial phenomenon at multiple points in space. For example, using the data from Figure 23.4, one could use a bivariate form of LISA to test *whether or not the HIV/AIDS distribution differs significantly from Year 1 to Year 5*.

23.6 THE FUTURE OF GIS

Because the current application of GIS tools is directly linked to computer technology, many techniques will expand along with the processing power of the PC and networks. One example of this is the growing list of available GIS applications and extensions mentioned previously. Many of these techniques were not readily available in the recent past because they rely on fast processors and can be memory intensive. Spatial clustering techniques such as the $Gi^*(d)$ statistic (when performed in ClusterSeer) rely on a Monte Carlo simulation to develop a measure of complete spatial randomness. This requires processing power only recently available at a capacity that could perform the analysis in a realistic period of time.

As the Internet becomes more available to rural areas and the developing world, web-based GIS will continue to expand, and is probably one of the fastest growing areas of GIS as far as map visualization and data entry. As mentioned previously, maps provide a powerful means of disseminating information. Web-based GIS is an excellent tool for mapping dynamic events such as disease outbreaks. In today's world of globalization, increased accessibility to transoceanic flights, and international trade of cargo, disease transmission of foreign diseases into naive populations is a reality. This creates the need for near real-time disease surveillance and map production to keep the population at large aware, if not safe, from disease risk. Because geographic information systems are based on current database applications, they are well suited to take center stage in disease surveillance and monitor programs. Additionally, as the examples from Hurricane Katrina have illustrated, GIS outputs can be developed quickly and can play an important role in emergency response. The advent of new tools such as Google Earth™ (http://earth.google.com) is quickly bringing the display of GIS data layers to non-GIS packages and non-GIS users. This will continue to expand and play a vital role in our understanding and sharing of disease data and surveillance well into the future. That stated, it is important that future GIS developers and spatial epidemiologists do not become inundated with technology or exciting computer techniques, but rather that they become rooted in the methodologies of GISc and epidemiology. The tools will only be useful if a fundamental knowledge of geographic thought and epidemiological principles are applied.

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ABBREVIATIONS

EOC: Emergency Operation Center
GIS: geographic information system
GISc: Geographic Information Science
GPS: Global positioning system
LSU: Louisiana State University
WHOCC: World Health Organization Collaborating Center for Remote Sensing and GIS for Public Health

GLOSSARY

Active Server Pages: A computer programming language that allows for the interactive editing of data values within a database through an Internet portal. This differs from the traditional Client–Server model of Internet hosting where an Internet user can browse the information of a server, but cannot edit it in any way.

Attribute: The defining characteristics assigned to a geospatial location within a GIS. These can be qualitative, categorical, ordinal, or continuous values within a GIS.

Batch process: A computer process by which a similar set of steps can be executed systematically until the operations have been performed on the entire data set.

Cold spot: An areal unit that lacks a significant number of disease cases surrounded by areal units with significant numbers of cases.

Coordinates: A pair of points (x,y) on a plain that when plotted simultaneously locate an event in that space. In the context of a GIS, these can be either geographic, such as latitude or longitude, or a pair of Cartesian coordinates specific to the study design.

Geocoding: Assigning data records from a database geospatial coordinates within a GIS environment, using one of several techniques for determining the correct position in space.

Geographic Information System: A combination of computer hardware, software, and a skill set of spatial analysis methods used to integrate geographic data and standard databasing techniques to develop spatial models and dynamic digital maps of primarily spatial data.

Georegistered: Meaning that a map, digital aerial photo, or satellite image has been registered (fitted) to a map projection within the GIS, so as to assign true geographic positions to locations on the map. The process by which digital imagery and scanned digital versions of paper maps are assigned geographic locations within a GIS.

Heads-up Digitizing: A method of point data entry into a GIS named for the manner in which the user interacts with a digital map on screen with eyes-looking-up and uses mouse clicks to assign spatial locations to specific events.

Hotspot: A signigicant cluster or spatial grouping of similar values. In this chapter, hotspots refer to geographic areas of high disease presence.

Raster data: A raster GIS uses a spatial grid with symmetrical cells to store the data. With raster data, attributes are assigned to each grid cell in the database and numerical values are used to represent various features.

Spatial precision: The horizontal resolution of location data on the ground. High precision data would be point data, individual locations for each event. Low resolution would be data aggregated to some areal unit, such as a political boundary or a grid surface.

Vector data: Vector GIS defines geography into three major objects: points (e.g., a disease case or an address), lines (e.g., the road connecting villages, a stream, a railroad line), and polygons (e.g., a political boundary, a building footprint, a water body). Descriptive data for each point, line, or polygon are stored as attributes in an accompanying data table that links each vector geometry with appropriate data values.

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CHAPTER 24

Vector Control by Surveillance Networks: The ECLAT Program and Chagas

J.-P. Dujardin[1] and C.J. Schofield[2]

[1] *Génétique et Evolution des Maladies Infectieuses, CNRS/IRD, Montpellier, France*

[2] *London School of Tropical Medicine and Hygiene*

24.1 INTRODUCTION

Chagas disease is a serious problem for public health in Latin America. In 1984, it was estimated that over 24 million people were infected [58], with a further 100 million people at risk. With extensive vector control programs during the last decade, these figures have been revised downward to around 12 million people infected [48], although large-scale control and surveillance programs are still lacking in several countries.

Chagas disease takes its name from Brazilian clinician Carlos Chagas, who first described it in 1909. It is a parasitic disease caused by *Trypanosoma cruzi* (Kinetoplastidae), producing a wide range of pathology from clinically undetectable lesions (60% of cases) to serious chronic problems of heart and digestive tract that can be fatal. Humans mainly become infected through contact with blood-sucking insects of the subfamily Triatominae (Hemiptera, Reduviidae) (see Fig. 24.1) those often colonize houses in rural areas [25]. Other mechanisms of infection include blood transfusion from infected donors, and occasional congenital transmission, oral contamination, or laboratory accident. Treatment (using benznidazole or nifurtimox) is effective only in the early acute stage of infection, where unfortunately the diagnostic is frequently overlooked, and frequently leads to undesirable side effects—especially in adults. The drugs are better tolerated in children, and treatment may be given to under-14s even in the chronic stage of infection, with the idea that this may impede the development of chronic lesions later in life [56]. Considered at the continental level, the debilitation caused by chronic infections makes Chagas disease one of the most expensive public health problems in the world in term of loss of productivity (expressed as disability-adjusted life-years) [60] although it is relatively simple to halt transmission by eliminating the domestic insect vectors.

In the absence of vaccine [9], and because of difficulties in curative treatment, control of Chagas disease relies primarily on measures directed against the insect vectors. These are large blood-sucking bugs (Triatominae) that are generally associated with small mammals, birds, or reptiles. But some species of Triatominae have become adapted to colonize human dwellings, where they feed predominantly on the people and their domestic animals. In houses, these bugs can develop abundant populations, often numbering several thousand individuals, causing significant nuisance to the householders and their animals. And because these, bugs can take substantial quantities of blood during each meal (up to 0.5 mL per meal for many adult Triatominae), they are also believed to contribute to chronic iron-deficiency anemia, as well as transmitting *T. cruzi* [41,49].

Elimination of domestic populations of Triatominae can generally be achieved by a thorough application of a modern pyrethroid insecticide (Table 24.1). If done on a small scale, however, the treated premises may be quickly reinfested by bugs accidentally carried in from untreated foci. For this reason, current control initiatives emphasize very wide area coverage—preferably over the entire geographical distribution of the target species, and generally involving several countries in

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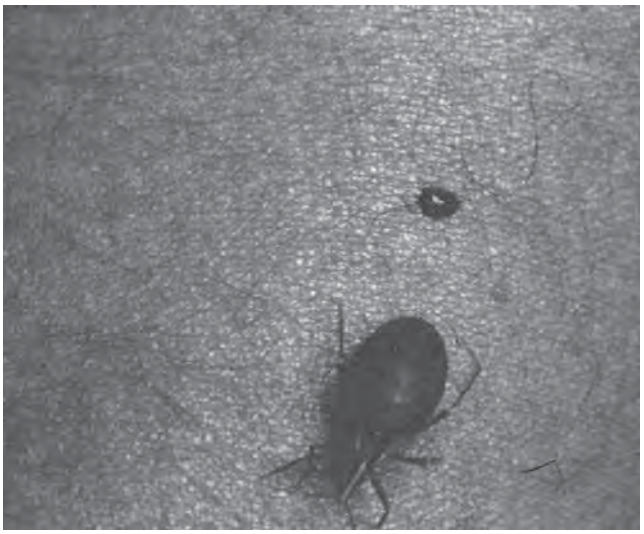

Fig. 24.1. Faeces of *Rhodnius prolixus* (here a fifth stage nymph of *R. prolixus*) left on the skin after feeding; if the bug is infected by *Trypanosoma cruzi*, this is the way the vector may infect its host: by depositing an infected drop of faeces. The parasite then enters actively through the mucosa or through the abrased skin.

a simultaneous campaign. For example, the Southern Cone Initiative launched in 1991 now covers the entire distribution of *Triatoma infestans* in Argentina, Bolivia, Brazil, Chile, Paraguay, southern Peru, and Uruguay. Similarly, the Central American Initiative launched in 1997 is primarily directed against *Rhodnius prolixus* in Guatemala, Honduras, El Salvador, and Nicaragua. In both these initiatives, the vector control measures—coupled with measures to improve blood transfusion control—have been highly successful, with Chagas disease transmission already interrupted in Uruguay, Chile, and large parts of Brazil, Argentina, Paraguay, Bolivia, and the Central American Countries [35,52]. Further regional initiatives are being developed for Mexico, Andean Pact countries, and the Amazon region. In all cases, however, it is recognized that extensive long-term vigilance will be required to avoid recolonization of dwellings in treated areas, either by the original species or by another species adapting from a silvatic habitat to colonize the houses. Here, we argue that epidemiological vigilance, when connected with international research, can be a fruitful activity on scientific ground with very positive public health output. The ECLAT network, by creating an international link between scientific research and operational activities, could maintain an efficient and continuous surveillance network during years. This partnership gave scientific research the opportunity to focus on relevant topics of immediate interest for public health, and it allowed health authorities to be aware of new techniques or new discoveries otherwise restricted to highly specialized literature. Its international nature also increased motivation and helped maintain continuity both of the research and surveillance activities.

TABLE 24.1. Pyrethroid Insecticides Used Against Domestic Triatominae

Insecticide	F	*R* (rate, mg.a.i./sq.m)	MS
Deltamethrin	SC	25	Aventis/Bayer
Lambda-cyhalothrin	WP	30	Zeneca/Syngenta
Cyfluthrin	WP	50	Bayer
Beta-cyfluthrin	SC	25	Bayer
Cypermethrin	WP	125	Various

F, formulation; *R*, recommended doses; MS, main supplier.

24.2 ORIGIN AND SPREAD OF HUMAN CHAGAS DISEASE

T. cruzi is a widespread parasite of small mammals in the Americas, with a distribution roughly from the great lakes of North America to southern Patagonia. Although it may originally have been transmitted directly between marsupials hosts (especially didelphid opossums) [51], it is now almost entirely transmitted by various species of Triatominae—especially those that colonize small mammals nests and marsupial lodges. Well over 130 species of Triatominae are now recognized. Most are of silvatic habit, but may occasionally fly into houses. Some also colonize peridomestic habitats such as chicken coops and goat corrals, and a few species colonize human dwellings—especially in rural areas. It is these "domestic" species—especially *T. infestans* in the Southern Cone Region, and *R. prolixus* in Colombia, Venezuela, and parts of Central America—that are of greatest public health importance and epidemiological significance as vectors of *T. cruzi* to humans.

Archaeological studies indicate the presence of *T. cruzi* in pre-Colombian mummies in the Andean region of South America (Chinchorro culture of northern Chile and southern Peru) dated up to 9000 years BP [5].

However, historical reconstruction suggests that the main expansion of human Chagas disease was in post-Colombian times, particularly during the last 150 years, associated with human migrations and accidental transport of "domesticated"[1] Triatominae [50].

Genetic studies on different geographical populations of the target vectors have confirmed the historical records suggesting their progressive but fast, recent spread largely due to human activities. These studies led to a predictive model explaining the spread of the human disease, that is, the birth of the disease itself [22]. Steps of this model are (i) adaptation of the insect to domestic conditions, (ii) passive dispersion of

[1] Synanthropic Triatominae are usually called "domestic" and/or "peridomestic" Triatominae by field entomologists; this expression will be used in this text

these domestic forms with humans, (iii) isolation from their original, wild foci, and (iv) lack of entomological surveillance or preemptive control response. In a few decades, this mechanism may contribute to the installation of a widely extended domestic vector, leading to widespread transmission of the disease [22]. Similar problems could arise with the same or with other vectors, to which the appropriate public health response is through an active surveillance network. To some extent, this surveillance requirement has been addressed by a combination of active surveillance by the public health authorities, coupled with community-based surveillance by the householders in endemic regions.

Fig. 24.2. Adult specimen of the main vector in the seven southernmost countries of Latin America: *Triatoma infestans*. (Photo by Marcia Gumiel, Bolivia.)

24.3 THE DISPERSAL OF THE MAIN VECTORS

Prior to the Southern Cone Initiative launched in 1991, primarily against *T. infestans*, this species was considered responsible for more than half of all transmission of Chagas disease to humans—responsible for infecting some 12 million people in the seven southernmost countries of Latin America: Argentina, Bolivia, Brazil, Chile, Paraguay, Peru (Arequipa Province), and Uruguay [25]. *T. infestans* is an efficient and widespread vector, extended over large geographic areas, highly dependent of domestic structures, but also found in silvatic habitats under rockpiles and parts of Central Bolivia [29]. Historical reconstruction suggests these silvatic populations first entered domestic habitats in pre-Colombian times—possibly associated with the hunting and domestication of its wild guinea pig hosts [50]. These domestic populations were then spread—probably by accidental carriage with migrating humans—mainly during the last century [13,31,34,38], reaching their maximum extension in northeastern Brazil just prior to 1981 [4,6,47]. In Central Brazil (states of Goias, Minas Gerais, and Bahia), *T. infestans* was unknown until the 1930s, but progressively replaced the local domestic vector—*Panstrongylus megistus*—that had originally been incriminated by Carlos Chagas two decades earlier. Genetic studies confirmed the scenario of a recent and rapid geographical spread of *T. infestans*, by showing low genetic heterogeneity of its populations [27,28,32,36], and suggesting a cline of decreasing variability from Bolivia to other countries [26]. Together with these genetic studies, the presence of silvatic foci only in Bolivia strongly suggested a Bolivian origin [29]. Further cytogenetic studies now suggest this expansion was completed in a two steps process, a first Andean expansion, followed later by a lowland expansion probably contemporary to Spanish conquest [39].

In Venezuela, Colombia, and parts of Central America, the most important vector of Chagas disease is *R. prolixus* [25]. Again, historical reconstruction and genetic comparisons of different populations suggest a relatively recent spread from an original domestic focus probably in Venezuela [17,55]. The spread of *R. prolixus* into Central Colombia may have been in association with Spanish expeditions from Venezuela in the sixteenth century. However, its spread into Central America seems to have been much more recent and possibly due to an escape from a laboratory colony in San Salvador in 1913 [23]. By 1915, *R. prolixus* had spread into rural houses in El Salvador [37], and then into neighboring countries—reaching its maximum Central American distribution during the 1950s. This included parts of Southern Mexico, Guatemala, Honduras, El Salvador, and Nicaragua [19]. These Central American populations of *R. prolixus* reached northern Costa Rica in 1953 (probably in association with migrant workers from Nicaragua) but were quickly eliminated by insecticide spraying [45]. Since then, its distribution has been progressively reduced, particularly as a result of concerted action through the Central American Initiative launched in 1997, and now includes just a few remaining foci in eastern Guatemala, Honduras, and western Nicaragua. Venezuelan populations of *R. prolixus* were also reduced through the national campaign (1966–1972), and some progress has been made in eliminating this species from houses in parts of central Colombia.

The distribution of other domestic Triatominae also seems to have been influenced by accidental carriage in association with human migrations. *T. dimidiata*, for example, is the second most important domestic vector of Chagas disease in southern Mexico and Central America, where it also maintains widespread silvatic populations. But in Ecuador and parts of northern Peru (Tumbes), *T. dimidiata* seems exclusively domestic and is the main vector in these regions. Again, historical reconstruction combined with genetic comparisons of different populations indicates that *T. dimidiata* was first domesticated in the Tehuantepec region of Central America, and that a subset of these domestic populations was then accidentally carried to the port of Guayaquil in Ecuador following well-established pre-Colombian maritime trade routes [1]. A more recent example concerns *Rhodnius ecuadoriensis*—of little epidemiological significance in its native Ecuador where it primarily inhabits palm tree crowns, but a major domestic vector in parts of northern Peru (La Libertad) where it is exclusively domestic. In this case—although the evidence

requires further confirmation—it is suspected that *R. ecuadoriensis* has been accidentally brought from Ecuador to Peru in lorries returning after delivering the Peruvian grape harvest [55].

Parallels in the adaptive history of these important vector species support the idea of a common strategy for their control. Silvatic populations of these species can be considered to have a "natural range"—albeit probably mediated by passive carriage with their silvatic vertebrate hosts. But an original domestication event, followed by dispersal of the domestic populations in association with human movements, has led to domestic populations of these species occurring well outside their original range—and it is these domestic populations that are of greatest epidemiological significance as vectors of Chagas disease to humans. Moreover, this process seems to involve genetic bottlenecks, founder effects and genetic drift [22], together with selection for the optimum genotypes for domestic habitats [54], so that the domestic populations tend to be of reduced genetic variability and consequently more vulnerable to available control methods. The combination of feasibility to eliminate domestic populations with the idea of "rectifying" the accidental transport of these populations outside their original range forms a compelling argument in favor of large-scale elimination of these domestic Triatominae. However, the capacity for domestication and passive dispersal may be similarly shared by many other species of Triatominae. The resulting working hypothesis is that passive and important migrations outside the current range of its original, wild foci, would be a trait specific to any other species becoming highly dependent to human environment [25].

24.4 FROM DISEASE TO PUBLIC HEALTH PROBLEM

24.4.1 The Nature of the Disease

As a human suffering, Chagas disease is not well known, and is presently classified as "neglected disease" by the European Commission. The acute phase of the disease lasts a few weeks, corresponding to the diffusion of the parasite into blood circulation and its entry to muscular cells, frequently cardiac cells, and hearth RX may show enlargement of the organ. Although this acute phase may be lethal, it generally happens without serious consequences, often being comparable to a bad cold. During the chronic phase, however, and after a few decades, up to 40% of patients may develop a severe disease. A proportion of them suffers from cardiopathy ranging from arrythmias to complete bundle-branch blocks requiring pacemakers implant. Cardiac aneurysm can also occur, leading to cardiac rupture (sudden dead syndrome) on exercise. Another proportion of infected people may develop megaorgans of the digestive tract, with intestinal peristaltism interrupted in severe cases, leading to difficulties in swallowing in the case of megaesophagus, and inability of stool transit in the case of megacolon.

The clinical outcome of Chagas disease, asymptomatic or lethal, heart or digestive disease, remains unpredictable. Geographic variation is obvious [43]. It has been attributed to differences in human genotypes and to differences in parasite genotype [3,57], virulence, growth rate, and tissue tropisms. In genetic terms, there appears to be two main lineages of *T. cruzi*—now denoted *cruzi* 1 and *cruzi* 2—that can be characterized by isoenzymes and DNA sequence differences. Of these, *cruzi* 1 seems more homogeneous and has been regarded as the more primitive form [51]; in humans, *cruzi* 1 tends to be associated with cardiac lesions, but rarely—if ever—with the lesions and dilations of the digestive tract known as "megas." By contrast, *cruzi* 2 is more heterogeneous, and although infections with forms of *cruzi* 2 are also typically associated with cardiac lesions, they are also frequently associated with megas (particularly megaesophagus and megacolon) especially in the Southern Cone countries. In neither case, however, is the pathogenesis clearly understood [10,42,44], although the cardiac lesions are often attributed to parasite stimulation of a host autoimmune response, and the digestive tract lesions are often attributed to progressive neuronal destruction of the peripheral sympathetic nervous system.

As a research topic, the pathogenesis of Chagas disease remains a major challenge [40],—especially in understanding prognosis and determining which factors will contribute to the development of chronic lesions. One feature that has recently come to light is that the vector control programs themselves may have an impact in progression of the disease—even amongst those people already infected [21]. Since the launch of the Southern Cone Initiative against Chagas disease in 1991, clinicians report an apparent decline in the average severity of chronic lesions amongst those infected prior to the vector control campaign. This could suggest that a reduction in the rate of reinfection of chronically infected people (due to elimination of the insect vector) has contributed to a reduction in the severity of their disease—an idea subsequently supported by experimental studies of reinfections in mice, and already suspected by clinicians [18].

24.4.2 The Disease of Poverty

As for many parasitic diseases, Chagas disease mainly affects people of low economic status, and there is a broad correlation between the occurrence of the disease and poor quality housing [12]. Traditional rural dwellings of earth, sticks and palm thatch, generally provide suitable shelter for domestic Triatominae, and rural houses are often close to silvatic foci of these bugs, so that they tend to be the first to be colonized. But even for human dwellings with higher standards of construction, the vectors easily colonies chicken houses and other domestic animals enclosures. With the development of large cities with surrounding precarious constructions, Chagas disease also became periurban in many Latin American countries [14].

Actually, even cities are not protected against some vectors [8,30,46]. Not only the habitat provides shelter to the insect

Fig. 24.3. Rural house in the Chilean Andes, infested with *Triatoma infestans* (Fig. 24.24.2).

Fig. 24.5. Typical house in Venezuela, infested with *Rhodnius prolixus*.

but also this latter has more or less preference for it, according to the species. Some authors have suggested classifying vector species of Triatominae according to their ability to colonize houses, going from completely silvatic to highly domestic species [50].

The importance of the domestic habitat in terms of vector colonization and transmission of Chagas disease has been well recognized—even since the earliest studies of Carlos Chagas himself [15,16]. And this has led to a number of calls—and many projects—to combat the disease through programs of improved rural housing. Such programs have met with various levels of success [11,53] but all have been limited in scale both by availability of funds and by different levels of community acceptance. House improvement programs tend to be considerably more expensive than vector elimination by insecticide spraying, and the proposed improvements do not always meet with the approval of local communities. They are also relatively slow to implement, and often require a higher level of subsequent maintenance than the local communities are able to carry out. There are also practical difficulties in the sense that house improvements alone are rarely sufficient to extinguish an existing infestation with Triatominae, and ethical considerations in the sense that presence of domestic Triatominae should not be the primary criterion for house improvement (because this may disfavor poor families that do not have domestic Triatominae). It is generally recommended, therefore, that rural house improvement should be considered an independent developmental goal, irrespective of the presence or absence of domestic Triatominae, whereas programs to eliminate these insects should proceed independently.

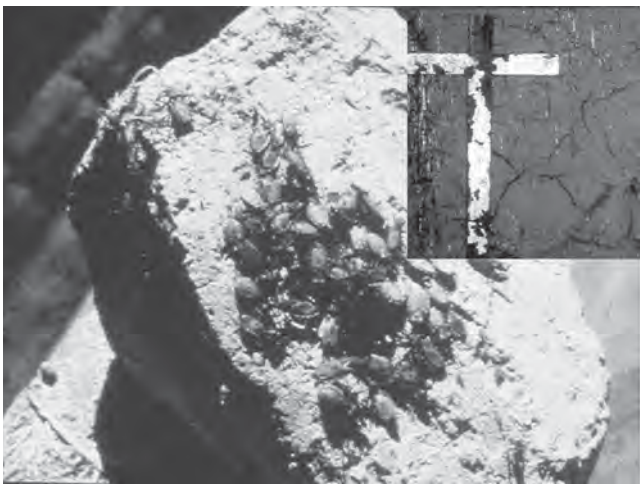

Fig. 24.4. One stone from the house wall is turned over to reveal many *Triatoma infestans* adults and nymphs; top right: the faeces of *T. infestans* (Fig. 24.24.2) streak down the house wall.

24.4.3 Socioeconomic Impact

At a societal level, the social and economic importance of Chagas disease derives both from its high prevalence and the severity of its symptoms—especially amongst the most productive age-classes between 20 and 40 years. Overall prevalence rates of around 5–6% were typical of most endemic countries prior to the current large-scale control initiatives, although local prevalence rates often exceeded 50% in some areas. In the initial acute phase of infection, which can be fatal without treatment, the patient may be incapacitated for 2–3 weeks due to fever, diffuse chest pain, insomnia, and general discomfort. During the chronic phase, however, up to 40% of surviving patients become incapacitated because of either severe cardiopathy or digestive tract megasyndroms. Costs of diagnosis, clinical follow-up, and supportive treatment are high—over US$ 120 per year even in the case of clinically asymptomatic patients [7], but rising to several thousand dollars where cardiac pacemaker or corrective surgery are required. In Bolivia, even considering that only 10% of

infected people had access to medical care, USAID estimated the annual loss due to Chagas disease at 100 millions of US dollars—then over twice the national health budget for the country. Estimates by the World Bank (1993) prior to the current large-scale control initiatives, ranked Chagas disease as by far the most serious parasitic disease of the Americas—far outranking even the combined socioeconomic impact of other parasitic diseases such as malaria, schistosomiasis, and leishmaniasis [52]. At the level of the individual, however, the impact of the infection may be equally as severe as that of the disease. Diagnosis of a chronic infection—even if asymptomatic—can lead to severe stress and confusion. Some countries continue (by law) to refuse employment to those diagnosed as infected, even when no disease is apparent. Suicides have been reported, following such diagnosis, assumed due to the difficulties of reconciling the idea of living with an untreatable infection that may (or may not) develop into a life-threatening disease. In poorer rural communities, infection and incapacity of the head of the household can lead to hardship for the rest of the family. In parts of Central America prior to the current control initiatives, we have seen testaments from chronic Chagas disease cases bequeathing their pacemakers to the eldest son—presumably in the sad expectation that he too will develop a similar need in later life.

24.5 CONTROL AND SURVEILLANCE

24.5.1 Control Strategies

The essential rationale of Chagas disease control is to halt transmission, and to provide treatment and support for those already infected. Interruption of transmission includes serological screening of blood donors to reduce the risk of transfusional transmission, but relies most heavily on elimination of domestic populations of the Triatominae vectors. As with other vector disease vectors, control of Triatominae on a small scale is difficult to sustain due to reinvasion of vectors from untreated foci. This sets the increasingly perceived need for large-scale initiatives and, because it is difficult to maintain control interventions indefinitely, there is also a need to design strategies that can reach a sustainable end point [33]. In the case of Chagas disease, the control strategy is, therefore, based on elimination of existing domestic vector populations—generally achieved through a single thorough insecticide application in each infested house (Table 24.1)—followed by continuous vigilance both by the local communities and by the public health services, with selective interventions wherever new domestic infestations are suspected. This basic control strategy is now being progressively implemented through large-scale initiatives involving most countries of Latin America.

The essential control strategy is focused on the elimination of all domestic populations of the insect vectors, and prevention of recolonization by (a) eliminating neighboring domestic and peridomestic colonies that could serve as foci for reinfestation, and (b) sustained entomological vigilance coupled with selective retreatment of houses if recolonization is detected. For operational purposes, the control programs work within the context of existing administrative units (localities or villages, within municipalities/departments/veredas—according to local administrative systems). Each locality is first mapped to establish the number and distribution of houses, with a search of each house to establish whether or not the target vectors are present. Where the target vector species is considered a feasible target for local elimination—as in the case of *T. infestans* in the Southern Cone countries, or *R. prolixus* in Central America—then all houses of each locality where one or more of these insects is detected will be sprayed, regardless of whether an individual house is infested or not. The reasoning is that because available sampling methods are imprecise, it is considered more effective to spray houses even if they are not actually infested, rather than risk not spraying a house when it is in fact infested. For other vector species of Triatominae, the decision to spray all houses, or only those that are apparently infested, is taken on the basis of the overall house infestation rate for that locality. In the case of *T. brasiliensis* in northeastern Brazil, for example, all houses are sprayed in localities showing 20% house infestation rates or more, whereas in localities with less than 20% house infestation rates spraying is confined only to those houses where infestation is confirmed. This survey and spraying cycle is generally repeated at 6-monthly or annual intervals, until all houses in the locality appear uninfested and that locality is then declared to be under the vigilance phase.

24.5.2 Vigilance Strategies

Vigilance, or epidemiological surveillance, represents a series of measures designed to detect—and respray if necessary—any new or surviving colonization of houses by vector Triatominae. In very general terms, this may involve house surveys by trained personnel of the operational vector control services (sometimes known as "active surveillance") and/or community-based vigilance whereby householders are trained and requested to report the finding of any bugs in

Fig. 24.6. In Paraguay, after the vector control program, this family can now sleep peacefully, without being bitten by Triatominae.

their houses. Several "tools" are available to assist in this, including visual inspection of the house structure (using a torch to see into cracks, and long blunt forceps to withdraw any bugs found), spraying with irritant nonresidual pyrethroids (e.g., 0.2% aqueous tetramethrin) to dislodge any bugs that may be present, artificial refuges pinned to the house walls where bugs may collect, sheets of white paper, or calendars pinned to the house walls that may reveal streaks of recent bug feces, and householder information notices together with a self-sealing plastic bag in which householders can put any bugs they encounter. In addition, health education is now widely offered in schools, clinics, and others, to help ensure that householders are aware of Triatominae and Chagas disease, and willing to help in the community-based vigilance. In most cases, active vigilance is maintained for the first year following the initial control interventions, but progressively greater reliance is then given to community-based vigilance. This can be effective, but requires continual reinforcement (e.g., periodic community discussions) and a well-organized system of response to any notifications of possible new infestations. Communities that feel their notifications are ignored can quickly loose interest in collaborating with the public health services.

24.6 VIGILANCE AND RESEARCH

24.6.1 Research and Vigilance

For much of the last century, research on Chagas disease was focused on understanding the epidemiological problem and seeking ways to control the domestic vectors. These aspects are now sufficiently understood to provide the social and biological rationale for large-scale control interventions, together with demonstrable evidence of the success of well-organized interventions—both for blood-donor screening and for halting transmission by elimination of domestic vector populations. The research focus is, therefore, changing to concentrate much more on epidemiological and entomological vigilance, especially factors that influence vector domestication, and ways to sustain adequate vigilance in the face of changing epidemiological patterns. This requires a much greater understanding of Triatominae populations that currently have silvatic habits, but may—under certain circumstances—invade domestic premises and potentially form new domestic colonies.

24.6.2 Endangered Continuity

In many countries, especially in parts of the Southern Cone Region and Central America, the success of the vector control interventions has led to a decline in political priority for these interventions. This apparent paradox—known in Spanish as "el castigo del éxito" (lit: the punishment of success, i.e., self-defeating success)—derives from the widespread view that resource allocation for public health should be proportional to the perceived severity of the problem. Thus, where successful control interventions reduce this perceived severity, then the premature assumption is made that the problem no longer requires continuing investment. Such a view risks future recrudescence of transmission, and takes no account of the marginal costs of reaching a sustainable end point after the initially successful interventions [2,21]. Moreover, with the ideological drive to decentralized public health services, irrespective of the biological and operational constraints of particular epidemiological patterns, it becomes even more urgent to conceptualize a future scenario in which disease transmission can be maintained at levels low enough to be compatible with projected health service capacity, but also with adequate provision for any new cases that may occur. For Chagas disease, control has long been conceptualized on the basis of intervention followed by various levels of surveillance (also known as "vigilance"), in which the most successful long-term programs have progressively adapted the surveillance strategy in accordance with changing epidemiological patterns [59].

24.6.3 The Role of Research

Even with progressive adaptations, long-term epidemiological surveillance becomes unsustainable unless it can be integrated with other community health care activities, which in turn risks loosing the specialist expertise required, for example, in vector and parasite identification, and interpretation of epidemiological patterns and trends. Yet that specialist expertise is present, continually developing and, to a large extent, independently financed—in the research community. Potentially, therefore, it is to the benefit of all to develop closer links between the executive health services and the research community, such that the research becomes more focused on epidemiological surveillance, and the results of that research can be more readily assimilated within the health services themselves. The need for research to sustain the control initiatives has been well recognized—partly to provide the medical, social, and biological rationale underlying the political decisions, but also to interpret the changing epidemiological patterns and help to resolve operational problems. In Brazil, the research community played a key role in providing the social and biological arguments that prompted the first national campaign against Chagas disease during the 1980s [24,52].

24.6.4 The ECLAT Network

During the Southern Cone Initiative, the Andean Pact Initiative and the Central America Initiative, surveillance activities have been backed by development of the ECLAT network as a consortium of entomologists, geneticists, and control service personnel linked to the monitoring activities of the public health authorities, including preemptive studies of candidate or potential new domestic vectors. Through this mechanism, coordinated investigation projects were set up in almost every country of Latin America in collaboration with local health authorities, in a way that enabled a wide range of analytical techniques to be applied over the entire geographic range of each vector species. Network workshops provided

a forum for discussion of results with scientists and decision makers at the highest national level, as well as international observers. These contacts allowed scientists to turn their curiosity to some problems identified by health authorities, and these later were informed about new advances in the entomological or epidemiological knowledge. These meetings always gave rise to new research and collaborative projects, stimulating and improving what would have been otherwise only routine activities. Analysis of this paradigm—linking research and public health interventions—shows that it has worked well both to promote the control interventions themselves [20,21], and also to promote the essential research. At the international level, the research network has also helped provide greater continuity of action—even if only because the research community is generally less subject to the political changes that affect Health Ministry personnel and policies. But integration of similar networks at national levels has yet to be fully developed. In many countries, national research activities are largely independent of the national public health intervention services, with their results presented in scientific congresses and journals—rather than being also accepted as an integral part of the public health service itself. So a national research activity may reach the international scientific media, and only from there—sometimes—become incorporated back into the national public health services.

24.6.5 The ECLAT Lesson

From our experience (the authors are joint coordinators of the ECLAT network), we deduce a need within each endemic country to develop greater integration between the research scientists working on Chagas disease and its vectors, and the public health services with responsibility for Chagas disease surveillance and control. We base this conclusion on the following logic:

1. Chagas disease can be controlled by elimination of domestic vector populations using currently available techniques.
2. It is appropriate to do this, on medical, social, and economic grounds, in the sense that such interventions have high cost–benefit ratios, and high social value.
3. It is also appropriate to do this because it is biologically and operationally feasible to do so.
4. Successful control interventions will inevitably lead to a decline in resource allocation for Chagas disease surveillance and control.
5. Surveillance procedures focused specifically on Chagas disease vectors can be progressively adapted over the short to medium term, in accordance with changing epidemiological patterns. However, the quality of such surveillance will inevitably decline with success (i.e., declining likelihood of detecting new domestic populations) and also due to declining resource allocation.
6. The risk of new domestic infestations will remain as long as there are silvatic species of Triatominae that may adapt to domestic conditions; but this adaptation represents an intriguing challenge for research scientists.
7. New domestic adaptations may become of continental health importance if the domestic species is (passively) spread over large geographical areas, as it probably has been the case for the present main targets (*T. infestans*, *R. prolixus*, and *T. dimidiata*).
8. Research on silvatic Triatominae can be promoted by the need for surveillance, and may be independently financed through research grants.
9. Scientists involved with such research are generally less subject to political changes, compared to their counterparts in Health Ministries, and may, therefore, provide greater continuity of investigation.
10. Research on silvatic Triatominae should, therefore, be closely linked with the public health authorities, and conceived as an independent but necessary component of national epidemiological surveillance.
11. National research councils and international research funding organizations should, therefore, seek to promote greater integration—and possible cofinancing—of research and surveillance activities in areas where Chagas disease transmission remains a potential threat.

24.7 CONCLUSION

For Chagas disease control and surveillance, we may conceptualize an end point at which existing domestic infestations of Triatominae have been eliminated. Such an end point would not be universally sustainable, except with a high degree of entomological surveillance coupled with selective interventions wherever necessary. But such surveillance would itself be unsustainable if successful, and so would tend to decline in interest, quality, and resource allocation. We propose, therefore, that the national scientific community be encouraged to play a closer role in entomological surveillance, providing continuity and detailed focal studies, together with a degree of interpretation from which additional surveys and interventions would be proposed where and when necessary.

We must recognize, however, that although this end point may include elimination of many populations of Triatominae, it cannot contemplate eradication either of all species of Triatominae or of the causative agent—*T. cruzi*. The parasite and its silvatic vectors will continue to exist throughout the Americas, and this may lead to occasional contact with humans and transmission of a new case of human infection. This component of our proposed end point has been termed—The Acapulco Syndrome—[24], whereby occasional transmission can be expected due to adventitious silvatic bugs that occasionally enter houses but do not establish domestic colonies. In such situations, vector control becomes largely irrelevant, and the main surveillance imperative rests with the clinical health services, because of the need for swift parasitological diagnosis and treatment of the acute infection.

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CHAPTER 25

Contributions of Morphometrics to Medical Entomology

J.-P. Dujardin[1] and D.E. Slice[2]

[1]*Génétique et Evolution des Maladies Infectieuses, UMR CNRS/IRD, Montpellier, France*

[2] *Institute for Anthropology, University of Vienna, Vienna, Austria*

"J'ai besoin de savoir que tout n'est pas confondu. (I need to know that everything is not confounded)"

—Jean Tardieu, in La Part de l'Ombre

25.1 INTRODUCTION

Morphometrics quantitatively describes the morphological variation of objects. When applied to biological forms, it is a particular field of biometrics. In medical entomology, where a major interest is the biology of insects in their natural environment, morphometrics might be considered as a tool for quantifying the phenotypic variation of an organism. Morphometrics focuses on variation, its parameterization, and relation to extrinsic factors. As long as phenotypic variation has environmental and/or genetic causes, morphometrics can help detect local adaptations and genetic divergence among populations. Morphometric characters are related to growth and development, and they are usually continuous. Traditionally, they were estimates of distances between anatomical points called landmarks. More recently, they have come to be the coordinates of these landmarks in a given system of orthogonal axes. We will present here some concepts and statistical analyses related to the use of these data, insisting on their biological relevance, with some examples of applications in medical entomology. Both traditional and geometric approaches will be presented. Special attention is given to applications involving Triatominae (Hemiptera: Reduviidae), the vectors of Chagas disease in Latin America (see chapter in this book) and Phlebotominae, the vectors of leishmaniasis. Finally, some information will be given about morphometric software.

25.1.1 From Dimensions to Biology

In the absence of artifactual variation, a distance between two anatomical landmarks or their relative position to other such points (see Figs. 25.1 and 25.2), depend on the morphological development of the organism under study; their variation with geography is arguably an effect of both environmental influence and adaptive changes; and their changes from one species to another reflect the process of natural evolution. When properly analyzed, metric-trait variation allows one to read some biological and evolutionary information embedded in the morphology [79,81,91]. One of the earliest morphometric studies is illustrative. After a severe storm in February 1898, among the moribund sparrows taken to the laboratory by Bumpus, some survived, others died. Examining a few measurements of their skeleton, Bumpus showed that "the birds which perished, perished not through accident, but because they were physically disqualified," and "the birds which survived, survived because they possessed certain physical characters" [64]. Thus, a simple set of measurements was able to illustrate the Darwinian concept of selection for the most fit. Morphometrics has this ability to make visible to us many aspects of the biology of an organism, such as its physiology, its pathology, and its phenotypic or genetic evolution.

25.1.2 Tradition and Modernity

The virtues of traditional morphometrics are today improved by the introduction of geometric techniques [83]. Morphometrics is often presented as "traditional," making

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

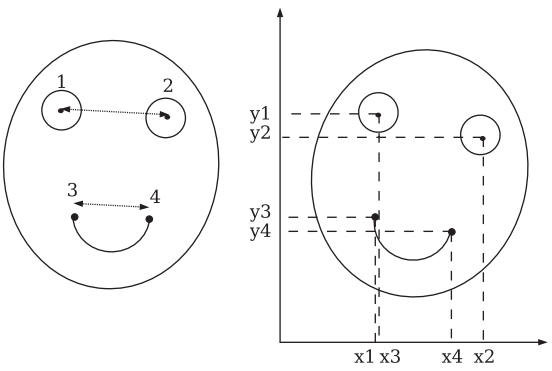

Fig. 25.1. Distances between anatomical landmarks (left) are advantageously replaced by coordinates of these landmarks in a given system of orthogonal axes (right).

use of limited sets of measurements, or "modern" (or "geometric"), making use of total geometric information (see Figs. 25.1 and 25.2). This difference, which derives mainly from the kind of metric data (distances versus coordinates), has generated a "revolution" [3,83]. Improvements or novelties exist indeed at various levels, the most important one being the direct description of shape itself. After some mathematical processing, the geometric figures represented by the landmarks are compared as different point sets between individuals or populations. Shape can also be studied by other direct techniques exploring outlines, textures, surface patterning, or even internal configuration of a form: These techniques will not be discussed in this chapter.

In spite of these advantages and the attractiveness of modern morphometrics, it has yet to gain popularity in medical entomology, where traditions may be hard to move.

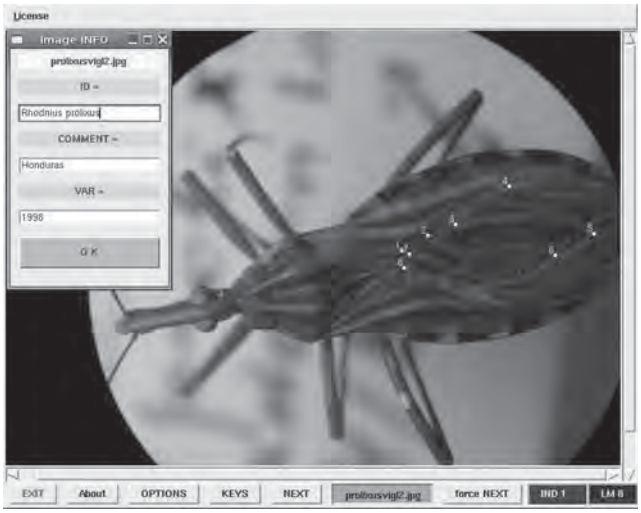

Fig. 25.2. Screenshot of a landmark collection session under the COO program (http://www.mpl.ird.fr/morphometrics). Top left window figures a small database gathering relevant informations. Yellow dots on the insect are the landmarks of the wing, labeled in the order of collection. The bug is an undissected, dry pinned *Rhodnius prolixus*. It is a South and Central American species of the subfamily Triatominae (Hemiptera, Reduviidae). After *Triatoma infestans* (see Fig. 25.12), it is the main vector of *Trypanosoma cruzi*, the causative agent of Chagas disease. The collection of landmarks is performed on a digital picture, using the "mouse-clic" with a dedicated program (here COO, see http://www.mpl.ird.fr/morphometrics; a more versatile and frequently used program is TPSdig, see http://life.bio.sunysb.edu/morph/).

25.2 CAUSES OF METRIC VARIATION?

Morphometric variation is under the influence of physiological (or pathological) status, adaptive changes, and genetic differences. Whereas different molecular markers applied to an insect will be differently affected by the environment—and some could be completely neutral—the metric characters are generally supposed to be of both environmental and genetic origin. The drawback is that there is no magic science to make the correct partition between these ultimate causes of metric variation: The genetic make-up of a population or/and its environment. The cause of morphometric variation cannot be found in the metric variation itself, it has to be searched by other methods and may become the object of an inquiry, itself. It is, however, possible to obtain from the data some helpful insight. As a first step to remove heterogeneous environmental influences and focus more on genetic differences, one could rear a complete generation of various samples under the same laboratory conditions [16,19]. It is however important to take into account possible genetic drift effects (number of founders) or even microenvironmental influences within laboratory conditions. A more speculative approach to tentatively partition causes of metric variation is the separate analysis of size and shape, based on the idea that shape would have less environmental variance [32]. If the main interest is to focus on environmentally induced changes, the study of fluctuating asymmetry of bilateral characters is an elegant, but challenging, approach (see Fig. 25.12). Adapted methodology (the study of bilateral structures) and accurate statistical techniques exist to reveal these environmentally induced changes [74], which are now applied to geometric morphometrics [55,66,89].

25.2.1 Physiological Causes

The main cause of metric differences related to physiology is obviously differential growth, when this growth heterogeneity is of environmental origin. Depending on more or less favorable environmental conditions, and on aging in vertebrates, individuals may be more or less developed. For conspecific individuals, traditional morphometrics proposes a set of statistical methods to remove this effect of age or growth from their metric variation. Scaling for size is interesting when one wants to remove the effects of physiological differences and concentrate on other causes of intraspecific variation. In that case, the size estimator—the one that's effect

will be removed from the metric variation—should be constructed from the dimensions of the anatomical structure under study, not from an external indicator (weight, etc.). An external indicator of size is acceptable when the objective is to study the meaning of size variation itself. A complete review of these methods for traditional morphometrics may be found in Ref. [54].

25.2.2 Pathological Causes

Some mutation or toxin may affect the morphogenesis of some individuals. Morphometrics is not always required to detect such changes, as they generally produce obvious, visible deformations. Many times, pathological causes produce extreme individuals ("outliers"). They may be removed from the dataset or included provided more robust statistical techniques are used [75]. However, when the environmental aggression is directed at populations instead of individuals, and moderate in degree (insecticides at nonlethal doses), the morphological change may be more subtle. It may become visible when examining the range of variation at some characters, or the level of fluctuating asymmetry for bilateral traits [69].

25.2.3 Adaptive Causes

Adaptation to a different ecotope, or simply geographic adaptation, are the likely causes of phenetic changes as observed within a single species. Adaptive causes are of a genetic nature, but we make them distinct from genetic causes (see next section) by their trigger mechanisms. Although genetic causes are random mutations or even speciation events, adaptive causes are within-species differentiation induced by the interaction of genotypes with various environments. In each environment the best-suited phenotypes are selected. With time, the genotypes corresponding to the best-adapted phenotype are selected, creating genetic differentiation in the corresponding populations ("genetic assimilation," [95]). Beside this collection of genotypes induced by phenotypic preference, adaptive differences are supposed to be produced also by direct selection on genotypes. These kinds of metric differences observed between conspecific, geographic populations disappear slowly, or sometimes do not disappear at all even after many generations in laboratory [50]. Note that under the same laboratory environment, new differences may appear which were hidden by environmental compensations, or due to genetic drift occurring in laboratory after many generations [28]. Rearing the insects is not necessary to detect adaptive causes, they are suspected also when it is possible to observe residual, significant differences after correction for differential growth. Specialized statistical procedures may show that even after removing the effect attributable to growth heterogeneity, significant differences remain. These "allometry-free" shape differences (see Section 25.3.2.1) are not the signature of species differences, they may also be found among conspecific populations living in different eco-geographical regions [32]. However, the partition of shape and size may be regarded as an analytical process increasing our capacity to interpret metric variation.

25.2.4 Genetic Causes

Interspecific metric differences most probably have a genetic origin. The nature of these genetic differences is not within the scope of this introduction, but their effects on metric traits deserve some discussion here. Although there is no special metric feature marking the difference between species, this topic is influenced by common ideas found in the literature, not completely true and not completely false.

25.2.4.1 The amount of differences The level of interspecific differences is generally (much) higher than the corresponding intraspecific variation, even across geographic populations. This led some authors to look for "how much" species differ in general [93], but this needs to be addressed separately for each group [32,33].

25.2.4.2 The nature of differences As shape is supposed to have a larger genetic variance than size, it is generally believed that species differ not only by size but also, and probably more, by shape [5]. The same approach as for adaptive causes may be applied here, that is, the partition of size and shape. There is, however, no rigorous way to attribute residual metric differences (i.e., shape) to the speciation process instead of to locally adaptive causes, and again, other methods are needed to clarify the source of variation.

25.2.4.3 The localization of differences When metric differences are found in organs responsible for mechanical reproductive barriers, they may be given more evolutionary importance than differences located elsewhere. It is well known that sandfly species generally have different dimensions or shape in at least one piece of their complex genitalia [96].

25.2.4.4 The circumstances of the differences When groups compared are sympatric, size or shape differences are a good indicator, although not a proof of speciation [14,19,35].

25.3 SIZE AND SHAPE

The characters provided by morphometric analyses, either distances between landmarks (traditional morphometrics) or their coordinates (geometric morphometry), contain information on both size and shape. Imagine you describe two triangles by the length of the three sides, say 3,3,3 units for the first one, and 30,10,30 similar units for the second one: These values are immediately describing different sizes, small (3,3,3) and large (30,10,30), and altogether different shapes, an equilateral (3,3,3) and an isosceles (30,10,30) triangle . . . . For live organisms, the question has been: Can we compare just shape, thus removing the size differences, and alternatively, can we compare just size—by constructing

a size estimator (like here the sum of the sides of each triangle, for instance). Morphometricians not only want to find a general character of size (global size) to better focus on size variation but also to remove its effects from the metric variation so that residual variation then represents shape variation. In either case, it is necessary to construct an acceptable estimator of size.

25.3.1 The Search for a Global Estimator of Size

In morphometrics, one single individual may be described by many characters of different anatomical parts, so that the question arises how to construct a relevant measure for the study of global size variation.

25.3.1.1 Dimensionality To avoid the complexity of working with and, especially, interpreting many characters (multidimensionality), one dimension of the organism could be adopted as representing its "global size." For instance, it is generally considered that wing length in mosquito is an acceptable index of body size [61]. This could, however, suffer some exceptions or it could be different for other insects. To avoid never-ending discussions about size representativity of one particular character, one could include each measured character as a partial estimate of global size. The many dimensions would then be combined into one summary of size variation, that is, one variable. According to the way this combination is done, the global size variation that is captured includes (allometry) or not (isometry) the unique variation at the level of individual characters.

25.3.1.2 Isometric change of size The isometric estimator of global size describes changes of size that do not modify the proportions of the object. From one object to another, every character of the object is multiplied by the same value (Fig. 25.3). Isometric change is described by a single coefficient. It could be compared to the amplification or reduction made by a quality photocopier: The proportions of the object are intact, its global size is changed. Figure 25.3 shows an isometric change applied to an equilateral triangle, which remains geometrically an equilateral triangle. An example of isometric size variation is the one used to construct "log-shape ratios" [22] from distance measurements: This estimation of global size is simply the average of all the log-transformed measurements of one individual. Another example of such estimation, used in geometric morphometrics, is called the *centroid size* [9], "centroid" because its computation uses the geometric center of the configuration of landmarks. It is the square root of the sum of the squared distances (SS) of each landmark to the centroid (see Gower, 1971 in 80). In the case of small, circular variation at each landmark, this estimator of isometric change of size is not correlated to shape variation [9].

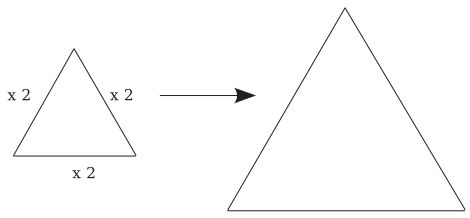

Fig. 25.3. An isometric change of size: proportions were left unchanged.

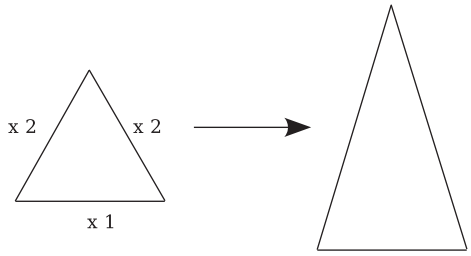

Fig. 25.4. An allometric effect: size and proportions have changed.

25.3.1.3 Allometric change of size With allometry, each body dimension has its own rate of growth. The allometric estimator of size variation takes this heterogeneity into account. Obviously, such complex change can alter the initial proportions (allometric effects). Figure 25.4 shows a simple allometric change applied to an equilateral triangle, which then became an isosceles triangle. The corresponding mathematical descriptor may use as many coefficients as characters. Various statistical techniques based on principal component analysis (PCA) are proposed to capture the allometric change as represented by the first principal component; in his review, Klingenberg [54] recommends the use of the first common principal component [4,44]. This approach is only valid when allometry is the cause of most variation in the sample, which is what the first principal component actually expresses. Allometric changes of size could be the effect of differential growth and age-related development, or, in case of various species, simply the effect of complex morphological evolution.

25.3.2 Shape As Size-Free Variation

The search for a good estimator of global size, either isometric or allometric, has two objectives: The first one is the study of size variation itself and the second is its removal from the metric variation in order to produce residual variables representing shape. Many statistical techniques have been proposed to produce variables independent of size variation, that is, size-free (shape) variables [54]. Because size has to be "removed" to represent shape, the accuracy of shape capture depends on the size descriptor, itself depending also on the characters used and their units, either the distances between anatomical landmarks (traditional morphometrics) or the coordinates of these landmarks (geometric morphometry). In both geometric and traditional morphometrics, the same concepts of shape construction are valid.

25.3.2.1 Allometry-free variables When it is the allometric component of size changes that is tentatively

removed, the residual variation should be called "allometry-free" shape variation. Statistical techniques to perform such scaling are often based on principal component analyses where each character is generally a log-transformed distance between anatomical points (traditional morphometrics) or coordinates (geometric morphometrics). Or, they may rely on techniques of regression or on additional information (for a review, see [54]).

25.3.2.2 Isometry-free variables When removing size variation focuses on the isometric changes, residual variation should be called "isometry-free" Proportions are preserved (see Fig. 25.3), so that "isometry-free" variation may be used to study data for which size has not been documented (see Figs. 25.5 and 25.6). The most common technique of size removal, including that used in modern geometric techniques, is to do just that: Factor out an isometric component of size from the metric variables producing, then, "isometry-free" variation.

25.3.2.2.1 Log-shape ratios In traditional morphometrics, log-transformed data are used [52] so that isometry-free variables are also called "log-shape ratios" [22]. Due to the loss of one degree of freedom, these variables still need an additional transformation through a simple principal component analysis, so that they can be subjected to standard statistical analyses [22].

25.3.2.2.2 Procrustes residuals The Procrustes[1] superposition refers to steps allowing the construction of size and shape variables from landmark coordinates for their use in morphometric studies. The reader interested in the full definition of the many specialized terms used in geometric morphometry will find a complete glossary at http://life.bio.sunysb.edu/morph/, which is an updated version of Slice et al. [88]. In the following steps, the homologous landmarks of various wings are superimposed on those of a consensus wing[2] so as to optimize some measure of goodness of fit—the minimum sum of squared distances of landmarks to their consensus homolog.

Translation First, each configuration of points is translated to the same centroid (Fig. 25.5)—a SS-minimizing step.

Scaling Second, each configuration is divided by its own centroid size, so that each configuration has a centroid size of one (Fig. 25.6). Size (isometric, see Section 25.3.1) variation is thus removed, or rather sequestered in a separate variable, centroid size, for additional study. This is not a SS-minimizing step, but results in all configurations being of a standard size [46,84].

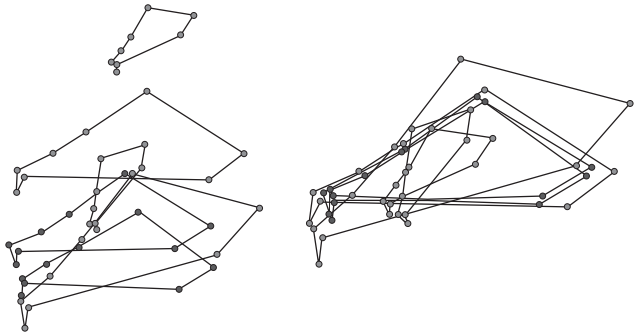

Fig. 25.5. This figure shows the effect of the translation step (left → right) for six configurations of eight wing landmarks (see Fig. 25.2) of the genus *Rhodnius* (Hemiptera, Reduviidae). The landmarks have been joined by line segments to aid visualization. Four wings belong to *R. robustus* (blue polygons, large ones from Venezuela, small ones from French Guyana), two wings are Venezuelan *R. prolixus* (brown polygons). The striking size differences apparent for the two *robustus* wings of Guyana are mainly due to different microscope magnifications. *Left*: Configurations of raw landmarks as they were captured on the computer screen: there are artifactual differences due to position (corrected by translation, see right part of the figure), magnification (later corrected by size scaling, see Fig. 25.6) and orientation (corrected after rotation, see Fig. 25.6). *Right*: One of the first steps in a Procrustes analysis is usually the translation of the configurations to a common centroid. MOG software, version 0.67 (Dujardin). See color plates.

Rotation Then, each configuration is rotated to fit the corresponding homologous landmarks of the consensus configuration (Fig. 25.6) using rotation parameters to minimize SS differences. Nonparametric techniques of fitting also exist, which might be more accurate when configurations are similar except at very few landmarks [80].

Residual Coordinates After Procrustes superimpositon is completed, coordinates have lost four degrees of freedom (for coordinates in a two-dimensional space) [53]. They could be used in the same way "log-shape ratios" are used, by performing a PCA on it and removing the noncontributive components. However, because their have a non-Euclidian metric (Procrustes distance), these residuals should, technically, not be used as input to perform multivariate analyses [87]. They can be used, however, in nonparametric tests as implemented in the Morpheus software (http://life.bio.sunysb.edu/morph/), see an example in Ref. [47].

25.3.3 Shape As Geometry

The specific contribution of geometric morphometrics is the construction of variables that describe the geometry of a configuration and can be used to describe differences

[1]Procrustes, whose name means "he who stretches," was a thief in Greek mythology (the myth of Theseus). He preyed on travelers along the road to Athens. He offered his victims hospitality on a magical bed that would fit any guest. As soon as the guest lay down Procrustes went to work upon him, either stretching the guest or cutting off his limbs to make him fit perfectly into the bed (Grose Educational Media, 1997–1998).

[2]In the most common case, Generalized Procrustes Analysis, the consensus is constructed using the average coordinates of individual landmarks in a sample. An initial specimen is chosen as a first approximation and the consensus is iteratively recomputed after subsequent fittings of the sample.

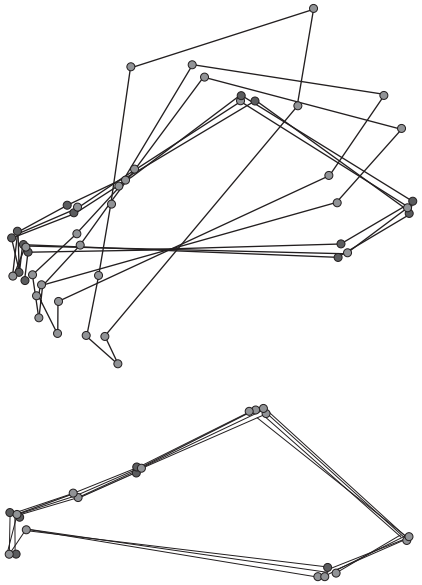

Fig. 25.6. Two more steps of the Procrustes superimpostion, the scaling (top) and the rotation (bottom) steps. *Top*: All configurations, after translation (see Fig. 25.5), have been scaled for size (the very small configurations seen Fig. 25.5 are now of equal size). *Bottom*: Objects are then rotated to allow the best superimposition possible across all landmarks. Consensus is not shown. Different statistical techniques exist for finding the best fit, the one most commonly used is based on the minimum Procrustes distance. Residual differences are size-free variation. MOG software, version 0.67 (Dujardin). See color plates.

between one configuration and another. After obtaining Procrustes residuals, an additional step allows the production of another set of variables called *partial warps*: They define the positional changes at each landmark in relation to a consensus or reference ordered by geometric scale. Partial warps are true *shape variables*, and have the properties of isometry-free variation. This geometric description of variation may still, however, contain allometric effects (the contribution of size variation to shape changes), and its construction relative to a reference means that shape variables depend on that reference. That is, the same individual may have different partial warps scores depending on the reference used, that is, according to the other individuals making part of the analysis. For a complete description of shape one must separately compute uniform (see below) terms (two for 2D data) [82].

25.3.3.1 Uniform and nonuniform components of shape The shape variables may be decomposed into two components called the *uniform* (or *affine*) and *nonuniform* (or *nonaffine*) *components*. These two components both describe a different kind of shape change relative to a reference object, one (affine, or uniform) is a global change that is the same everywhere, the other one (nonaffine or nonuniform) is made of locally distorting changes [9]. Figure 25.7 shows separate affine and nonaffine transformations with increasing size (affine transformations leave unchanged the parallelism of the grid, but see Ref. [8]). As these components are just the mathematical decomposition of shape variation, it is generally not advisable to analyze them separately [10].

25.3.3.2 Relative warps *Relative warps* are the principal components of the partial warps scores plus the uniform terms (they are produced by a simple principal component analysis). In comparing groups and/or visualizing group differences and patterns of variation, relative warps (or part of them) may be used as input for a discriminant analysis.

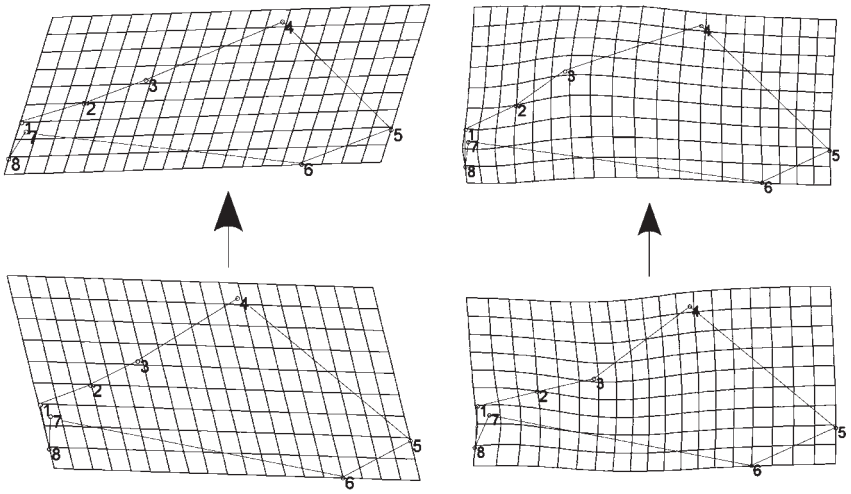

Fig. 25.7. Polygons connecting eight landmarks collected on the wing of *R. prolixus*, and inserted into a grid showing deformations from consensus. The figure illustrates the changes of shape due to growth heterogeneity among sympatric individuals (Pampanito, Venezuela), that is, it allows the visualization of allometry. Plots are thin-plate splines showing uniform (left) and nonuniform (right) changes from the consensus for the smallest (bottom) and the largest (top) wings. To help visualization, changes were amplified 10 times. tpsRelw software (Rohlf).

25.3.3.3 Thin-plate spline The thin-plate spline is a spatial interpolation function that can produce D'Arcy Thompson-like plots showing the geometry of shape changes between objects [9]. The TPSPLINE program [76], as well as the TPSRELW [77] or TPSREGR [78] programs, perform the necessary computations and plot the resulting differences as a transformation grid (see Fig. 25.7). Morpheus et al. [86] and Edgewarp [11] can compute both 2- and 3D thin-plate spline representations.

25.3.4 Which Shape?

When comparing samples of conspecific individuals, one usually wants to factor out exclusively allometric size to remove the effects of differential growth (or aging, in vertebrates). Indeed, among conspecific individuals living in sympatry, allometric changes are most probably attributable to growth heterogeneity. In case of significant residual variation after removing allometric changes, causes other than simple differential growth may be suspected. This conceptually interesting approach to explore intraspecific variation is limited by its own assumption of a common allometric axis, and more commonly applied in traditional morphometrics where this axis is expected to be linear. A common axis of growth, at least for the characters used, is not always verified (the NTSYS® software provides a relevant statistical procedure), and of course, less easy to find when populations are geographically distant, adapted to different environments, or actually when they belong to different species. In these situations where causes other than physiology are expected to play a role (genetic adaptation, microevolutionary trends, interspecific differences, etc.), scaling for isometric variation is a common practice in both traditional and geometric approaches. It is then recommended to verify (and quantify) the residual allometry by regressing shape onto centroid size.

25.4 MORPHOMETRICS AND MEDICAL ENTOMOLOGY

Medical entomology focuses on medically important insects. Most recent morphometric studies related to medical entomology have been dedicated to Triatominae and Phlebotominae.

Triatominae (Hemiptera: Reduviidae) constitute a small subfamily containing all the vectors of Chagas disease occurring in Latin America (see the corresponding chapter in this encyclopedia). They are large bugs (from one to four centimeters) with long generation times (a few months to more than 2 years, according to the species), and obligatory hematophages at all stages of their cycle, larvae or adults, males or females. Phlebotominae (Diptera: Psychodidae) are very small flies (less than 3 mm) transmitting *Leishmania* ssp., bacteria and viruses (see Fig. 25.8). As in mosquitoes, only females are hematophagous, they have short generation time (1 month), and cover cold and tropical areas worldwide. As both of these insects have medical importance, their correct identification is a crucial component of epidemiological study.

Fig. 25.8. Sandflies are very small, hematophagous Diptera (1–3 mm); their identification needs dissection, mounting and microscope amplification. Here we see the hyaline wing of *Lutzomyia* sp. (top), with parts of the thorax, abdomen, and genitalia (bottom) (from "Ciberatlas de los Flebotominae de Bolivia", Le Pont et al., see http://www.mpl.ird.fr/morphometrics).

Initially, morphometrics applied to medical entomology had the objective of aiding systematic research, so that it has been traditionally associated with description of species, often based on very few specimens [63,96] and described by a few ratios. This is unfortunate, because morphometrics is a population approach rather than a description of individuals. To be consistent, morphometric studies need more appropriate sampling techniques than used in taxonomy, and more adapted statistics than simple ratios. The use of simple ratios, although useful in some cases [45], is not a recommended practice [21], especially when used as a statistical technique to reduce the influence of size on shape [92]. As an illustration, see the obvious correlation of a head ratio with head size itself (Fig. 25.9), which is frequently used in the systematics of Triatominae. In medical entomology, ratios have been used as a taxonomic tool for decades; the first attempt to use multivariate techniques in removing size effects on a medically important insect is only a few years old [50].

The main interest of using morphometrics as a tool for species distinction in medical entomology is that it does not require the high level of entomological expertise to make an accurate species diagnosis in a specific group of insects. Health personal or nonspecialized laboratories could use similar concepts and techniques to classify insects in very different groups. This idea has been recently discussed for mites [7].

25.4.1 Systematics

25.4.1.1 Phlebotominae The taxonomy of Phlebotominae is still a very debated issue: This group of insects is huge (more than 1000 species [96]) and poorly understood. Cryptic species are not uncommon [15,34,59]. To help in distinguishing morphologically close species, multivariate

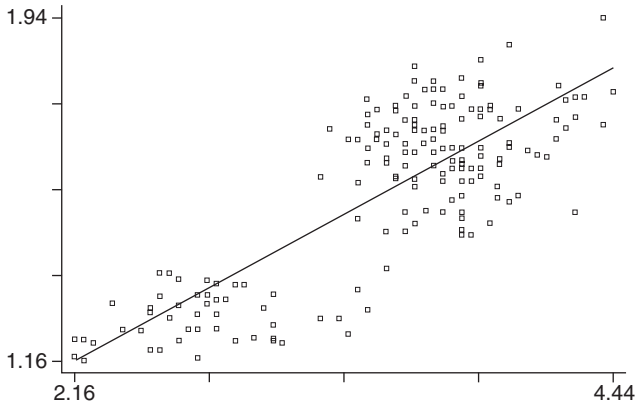

Fig. 25.9. The vertical axis shows the values of the AO/PO ratio in the genus *Rhodnius*: It is the ante-ocular distance (AO) of the head relative to the post-ocular one (PO). The horizontal axis presents the total length of the head in millimeters. This head ratio AO/PO is frequently used in the systematics of Triatominae, it is supposed to remove the influence of (head) size but, as shown here, it behaves as another estimator of size.

techniques on raw data have been suggested [58], and applied a few years later [1,18]. The partitioning of size-included and size-free variation has been successfully used to explore or detect hidden speciation [31,35,36,43], or as an additional taxonomic criteria in species description [60]. Very recently geometric morphometry has also been used, showing unexpected power in detecting cryptic species [56].

25.4.1.2 Triatominae There are less classification problems in the small group of Triatominae (137 species [41]). However, morphometrics can be particularly helpful in Triatominae systematics because these insects, unlike Phlebotominae, often lack discrete, qualitative characters allowing their discrimination (except color patterns) [63]. Multivariate techniques of size and shape partitioning were applied to one of the most confusing taxonomic problems in Triatominae: *R. robustus* and *R. prolixus* [49,70,72]. Between laboratory lines of these two taxa, that is, insects sharing the same environment during one generation after their field capture, allometry-free variation and geometric techniques disclosed consistent differences [94], allowing species distinction even on a single specimen [68].

25.4.2 Geographic Variation

25.4.2.1 Phlebotominae In sandflies, geographic variation of size is frequent and could interfere with species diagnostics [57]. The removal of size by means of multivariate techniques is intended to explore the stability of size-free metric properties. In a study covering 10 species of the genus *Lutzomyia*, it was shown that within large eco-geographical regions of South America, size-free variation was generally stable within species; however, this intraspecific stability was compromised when comparing samples from different ecological regions [32]. Geometric techniques seemed to provide more stable characters [33].

25.4.2.2 Triatominae Geographic studies partitioning size and shape (size-free variation) have been performed on a few species, and showed the influence of geography on metric properties [30,90]. A geographic study is expected to focus on conspecific populations, but the inclusion of another species may provide interesting taxonomic information. Two possibilities are (i) the within-species, geographic variation is lesser than interspecific variation, which is an expected feature or (ii) the geographic heterogeneity is wider and includes some other species. In this latter case, the study questions either the included species or the geographic populations themselves. For instance, in *T. dimidiata*, size-free divergence between cave populations of *T. dimidiata* and other populations of *T. dimidiata* were commensurate with interspecific metric distances either to *T. mexicana*, *T. pallidipennis* or *T. ryckmani* [13]. This too-wide geographic variation called for a taxonomic revision of *T. dimidiata*. Another example used landmark coordinates of the wings, showing that the controversial species *T. melanosoma* did not depart from the geographic variation of *T. infestans* [47]. This result, already disclosed by traditional morphometrics [40], did not support *T. melanosoma* as a different species, and was in agreement with genetic studies [71].

25.4.3 Comparisons of Morphometric with Genetic Variation

25.4.3.1 Phlebotominae Among closely related species, two studies have shown fair agreement between MLEE (multilocus enzyme electrophoresis) and morphometric variation [38,44]. Concordant results were also found for very distant, Old World and New World taxa (see Fig. 25.10) [37].

25.4.3.2 Triatominae The size-free variation of head and wings in the *Rhodnius* genus produced a classification in global agreement with phylogenetic reconstruction [29].

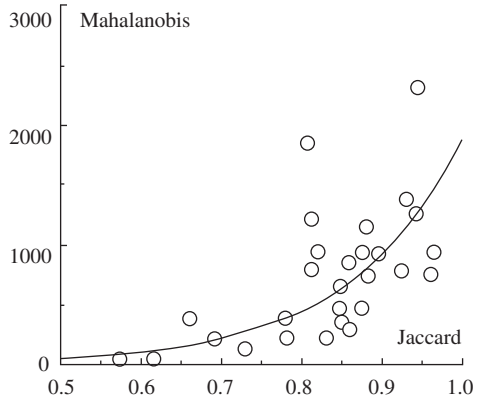

Fig. 25.10. Relationship between distances as computed from metric (vertical axis) and genetic (horizontal axis) characters, used to classify distant taxa of Old World and New World Phlebotominae (from Ref. [37]).

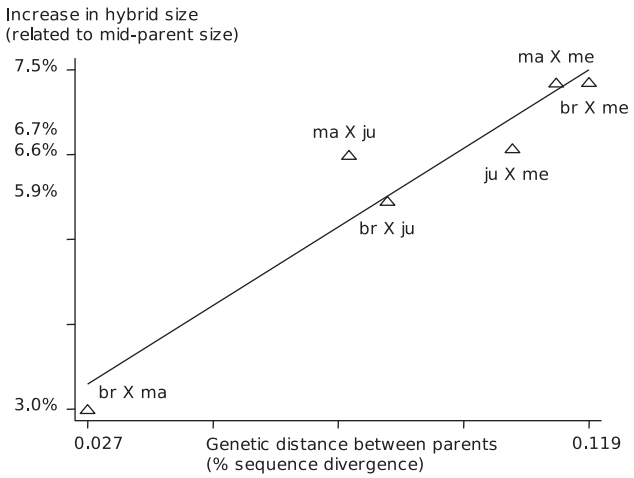

Fig. 25.11. Relationship between size in hybrids and genetic distances between parents; on vertical axis, the increasing of size relative to mid-parent size; on horizontal axis, the genetic distance between parents as inferred from mitochondrial DNA sequence; ma, macromelasoma; me, melanica; br, brasiliensis; ju, juazeirensis (from Costa et al., unpublished data).

A good fit between morphometric and evolutionary relationships was also found among close species in a small group of the genus *Triatoma* [73]. Such agreement between morphometrics and genetics has been verified in various instances in Triatominae [26,71,90,94]. Altogether, it seems that, at least for closely related species, there is a frequent accord between morphometric and genetic classifications [41]. Such agreement becomes higher within species, when comparing subspecies or geographic populations. Between seven laboratory colonies of *T. protracta* or between each of the five subspecies of *T. protracta*, each hybrid had an intermediate centroid size between parents (Dujardin et al., unpublished data). However, centroid size was increased in hybrids obtained from two other subspecies, *T. b. brasiliensis* and *T. b. juazeirensis*. It was larger than the mid-parent size, suggesting a heterosis, itself pointing to a probably consistent genetic divergence of parents (Costa et al., unpublished data). Extending this study to experimental hybrids among the four members of the Brasiliensis complex, it was possible to show a linear relationship between the genetic divergence of the parents [20] and the increase of size of their offspring (Fig. 25.11).

25.4.4 Topics Specific to Triatominae

In Triatominae, metric variation was also used to explore other topics, like their transition from sylvatic to laboratory or domestic environments, their reinfestation behavior, and their migrating history.

25.4.4.1 Adaptation to new ecotope The most epidemiologically meaningful niche adaptation of Triatominae is the transition from the natural, sylvatic to the domestic, or artificial environments [41,67] where the insect actually transmits the parasite to humans. It has been shown that the body is larger for specimens collected in natural conditions, versus their counterpart reared in laboratory [42,97]. Although the laboratory cannot be equated to domestic conditions of life, this decrease of size in artificial ecotopes seems to parallel a similar trend from sylvatic to domestic conditions [48,97]. Sylvatic specimens of *T. dimidiata* [97], *T. infestans* [25], *T. brasiliensis* [12], *Panstrongylus geniculatus* [51], and *P. megistus* [6] are larger than their domestic counterparts. This sometimes includes sexual size dimorphism. On average, females are larger than males [63], but old laboratory colonies of *R. neglectus* showed strongly reduced sexual head size dimorphism, and so did domestic populations of *T. infestans* relative to their silvatic counterparts [42]. Reduced sexual size dimorphism was not found however for head or wing dimension of a five-generation laboratory colony of *P. geniculatus* [51].

From natural to artificial ecotopes, after three to five laboratory generations, for head and wings dimensions in *T. infestans* and *R. stali*, there was not only a decrease of size but also a larger variance (Matias et al., unpublished data). In accord with this observation, fluctuating asymmetry was also shown to increase from sylvatic to domestic environments [40].

So far, all these changes were putatively related to environmental changes (the transition from one habitat to another), but these studies need to be refined by experimental work controlling some confounding variables like, for instance, blood source, insect density, or feeding patterns.

25.4.4.2 Migration history A cline of decreasing size also seems to follow the progression of past geographic migrations (generally passive migrations) of domestic vectors. It is apparent in *T. infestans* and in *R. prolixus* from their supposed original countries to the present periphery of their distribution: size decreases [39] and developmental instability increases [23]. It is indirectly supported in some cases where an insect is found in a limited area, outside its current territory (generally in domestic conditions only): These specimens are generally smaller than current representatives [2]. In such isolated or peripheral populations, metric changes and local adaptations [40] may even lead to undue new species description, see for instance *T. melanosoma* [17,47,62,71] or *P. herreri* (Gumiel et al., unpublished data) [65].

25.4.4.3 Reinfestation studies A few studies proposed morphometrics as helpful criteria for making decisions in entomological surveillance. Provided that samples were available from the population before insecticide application, relative metric similarities could suggest the origin of reinfestant specimens [27]. As a residual population is supposed to be the same generation as or the next generation to the individuals subjected to insecticide spraying, such an application is based on the supposition that an insect is more similar to its parents than to other insects. It is also based on the idea that insects reared in the same microenvironmental conditions (a few houses, a village) would share a significantly larger amount of metric similarity. Such idea would be less applicable to insects having

Fig. 25.12. Triatominae, or "kissing bugs," are generally large insects (1–4 cm). A North American species has been shown Figure 25.2. Here is an adult specimen of *T. infestans* (Hemiptera, Reduviidae), the main vector of Chagas disease in South America. As Hemiptera, it has two pairs of wings. The anterior pair is the best anatomical structure for morphometric studies: It is almost two-dimensional, so that the measurement error can be reduced, and it shows well-defined venations, so that homologous parts are easily recognized. In addition, these are bilateral structures, allowing studies about the symmetry of the insect (from the "Ciberatlas de los Triatominae," Dujardin and Matias, see http://www.mpl.ird.fr/ morphometrics).

short generation times (like Phlebotominae); its applicability actually depends on the stability of metric properties in one place from one generation to another, a feature that has to be explored in each case rather than theoretically predicted. This has been done for *T. protracta* by examining two successive generations of seven laboratory colonies: The geometry of the wing, male or female, was useful to assign any single specimen to its parents or close relatives in more than 90% of cases [24]. Thus, the geometry of the wing could be an interesting candidate to assess the origin of reinfesting specimens, either the descendants of previously killed bugs or immigrants from an external focus.

Traditional morphometrics (head measurements) already gave satisfactory results, as verified by genetic markers [26].

25.5 AUTHORS CONTRIBUTION TO MORPHOMETRICS SOFTWARE

With an intuitive understanding of multivariate and geometric analyses, it is often possible to perform valid morphometric studies thanks to specialized software, some of them freely available at http://life.bio.sunysb.edu/morph/ and http://www.mpl.ird.fr/morphometrics. We just mention here our contribution to this production.

25.5.1 Software for Multivariate Analyses

25.5.1.1 BAC BAC (for "Bootstraps, Analyses of principal Components"), beta version, multiplatform (Windows, freeBSD and Linux), presently commented in Spanish, performs different kinds of principal component analyses with limited graphical output. Assuming data are log-transformed measurements, BAC allows the use of bootstrapping to estimate the variation of allometric coefficients (eigenvector stability). After successive PCA using the same characters, BAC computes the angle between first principal components. Applied to *partial warps data*, BAC computes the corresponding relative warps.

25.5.1.2 PAD PAD (for Spanish words "Permutaciones, Analisis Discriminante"), multiplatform, beta version, performs a multivariate discriminant analysis (DA) with limited graphical display. For log-transformed data, if requested, PAD removes the isometric change of size, performs a PCA on isometry-free variables to produce isometry-free components, a representative set of which is then used for discriminant analysis. A similar approach is offered for *landmark-based* data. PAD allows random permutation of individuals among groups to test the significance of each pairwise Mahalanobis distance. PAD allows a reclassification of individuals, with and without cross validation. Supplementary data may be introduced.

25.5.2 Software for Landmark-Based Data Analyses

25.5.2.1 COO COO (for "Collection of Coordinates"), multiplatform (Windows, Linux) and beta version, is designed to collect coordinates of anatomical landmarks.

25.5.2.2 MOG MOG (for the Spanish words "Morfometria Geometrica"), multiplatform (Windows, Linux) and beta version, allows users to visualize the different steps of a Procrustes superimposition, generating Procrustes residuals, partial warps (shape variables) and centroid size for each individual. The output format is ready to use for multivariate analyses programs described above (BAC, PAD). Some of the illustrations used here were made with MOG.

25.5.3 Comprehensive Software

25.5.3.1 Morpheus et al. Morpheus et al. (Morpheus) is a program for morphometric research [86]. It supports a variety of data types, such as *n*-dimensional points, curves, images, user variables, and dynamic measurements. The program provides extensive import/export functions, including the estimation of landmark locations from truss distances, and advanced graphical capabilities for the visualization of morphometric data and results.

25.6 CONCLUSION

Morphometrics is a dynamic branch of biology. Technical and conceptual advances have considerably increased its resolution power. In medical entomology, it is no more a mere taxonomic tool, but it has also become a powerful way to explore intraspecific variation. Its relevance to quantify phenotypic changes makes it a valuable complement for biological studies on laboratory or natural populations, with possible applications to epidemiology. As long as phenotypic evolution is a relevant factor of speciation [85], morphometrics is one of the best quantitative approaches to evolutionary biology.

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CHAPTER 26

Surveillance of Vector-Borne Diseases Using Remotely Sensed Data

D.E. Gorla

Centro Regional de Investigaciones Científicas y Transferencia Tecnológica (CRILAR), Anillaco (La Rioja), Argentina

26.1 VECTOR-BORNE DISEASE SURVEILLANCE

Surveillance of a vector borne disease is the process of systematic collection, collation, and analysis of data with prompt dissemination to those who need to know, for relevant action to be taken [41]. It is a fundamental component of any program leading to the interruption or decrease of the vectorial transmission of a disease through the control and/or elimination of its vector. Surveillance systems aim basically at data collection and analysis to produce predictions either over time and/or space, to inform the health system about the potential of disease occurrence. As available resources for the health systems are frequently limited this information is crucial to stratify risk and determine where and when additional resources would be needed to maximize the effectiveness of field activities. Important as they are, surveillance systems are the Achilles heel of many vector control programs, especially in developing countries. Frequently, surveillance systems face problems associated with underfunding, organization, and continuity, leading to partial or complete failure of the control of vector-borne disease programs.

A number of surveillance systems associated with different vector-borne diseases have to deal with disease outbreaks, thus requiring tools to make predictions about when and where is the outbreak likely to occur. The further the forecast, the better the preparation of the health system [20]. In this kind of situation, the prediction tool will help the health system prepare in advance to cope with the epidemiological eventuality. Malaria, West Nile, and dengue outbreaks are typical examples. Outbreaks are associated with either the explosive increase of vector abundance and/or the triggering of pathogen circulation due to, for example, an unusual increase in temperature or rainfall.

Other vector-borne diseases do not produce outbreaks, but occur as endemic diseases over very large and sometimes unmapped areas. In these cases, the surveillance system has to make predictions to identify the areas with higher risk of disease transmission. In these situations, the predictions will help the health system to identify the areas most likely to be affected by the diseases. As the areas can be quite large, it pays having a tool with the ability to indicate where to assign resources for (as an example) the installation of a field site for long-term monitoring and/or to carry out a survey in order to identify infected people who could be cured with the existing treatments. Chagas disease in the Amazon region, sleeping sickness in Africa, and tick-borne disease are examples of these situations.

Important components of a vector-borne disease surveillance program, such us geographical coverage and sustainability over a long time, are rapidly affected by resource shortage and lack of organization. Within this context remotely sensed data recorded by earth observation satellites can help to improve the geographical coverage and sustainability of a vector-borne disease surveillance.

Satellite remote sensing, global connectivity, hardware power of desktop computers coupled with analytical power of computer software, particularly with the development of geographic information systems, lead to a strong increase of freely and widely available data on environmental and climate data, at various spatial and temporal resolutions, including some relatively long time series. Lack of disease data is nowadays a more common limiting factor than the lack of environmental information. Maintaining and strengthening disease surveillance

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

systems for monitoring the incidence of infectious disease, collecting high-quality, long-term disease data is essential for generating and refining models using remotely sensed data.

26.2 REMOTE SENSING AND VECTOR-BORNE DISEASES

The basis behind the use of satellite remote sensing for the study of vector-borne diseases relies on the fact that invertebrate vectors and the pathogens they transmit are affected by environmental variables that in turn can be estimated using satellite imagery. Sensors on board satellite platforms measure electromagnetic energy reflected or emitted from the earth surface. These measurements can be used to produce estimates of a number of meteorological and land cover variables. Among these variables, those related to climate (especially temperature, humidity, and rainfall) and measures of land cover are the most commonly used. A digital satellite image is a numeric grid that contains in each grid cell (= picture element = pixel) a value that represents the amount of energy that the sensor received from the earth surface. This numeric grid (arranged as columns and rows) is known as a "raster," one of the two formats used to represent spatial data (the other is the "vector" format described in detail in the chapter by Curtis et al. in this book). The data type contained in the image depends on the way the numbers are represented in each pixel. Usual datatypes are 8- or 16-bit images that define the file size and the number of different numbers that can be represented in a raster. An 8-bit image will contain numbers in the range 0–255 and will be smaller in size (for the same number of columns and rows) than a 16-bit image that can represent numbers in the range −32768 and +32767.

Depending on the sensor features, the image product might have more or less *spatial resolution* (pixel size may vary from one kilometer down to one meter), or *spectral resolution* (number of channels in which the electromagnetic energy received by the sensor is discriminated). Higher spatial and spectral resolution makes possible the discrimination of more details on the earth surface properties. Spatial and spectral resolution, together with the *temporal resolution* (frequency with which the satellite platform passes over the same point of the land surface; it is determined by the satellite orbit and can vary between twice a day to twice a month), defines the main features of the satellite image [14] (see Fig. 26.1).

The high spatial resolution of Landsat (pixel size of 30 m), Spot (10 m), and more recently Ikonos (1 m) and relatively good spectral resolution (2–8 channels) are ideal for local analysis and design of control activities. However, the temporal resolution of these products (16–26 days) reduces their value when the problem under study has a temporal change rate of the order of days rather than weeks, and/or the interest is posed on monitoring the seasonality of habitats that influences disease transmission variables. Frequently, and especially in tropical areas, cloud cover interferes with the use of images produced by the most commonly used *passive sensors* (like Landsat and Spot), that depend on the sun's energy reflected from the ground. Cloud interference can be avoided using *active sensors* (those based on radar technology that emits the energy that the sensor receives), a relatively common situation in tropical areas with high cloud coverage [14]. Alternatively, cloud contamination can be reduced using the method of maximum value composite (MVC) developed by Holben [16]. Essentially, the method uses a collection of images produced by the satellite sensor during a time period, for example, 10 daily images. Suppose the variable to be estimated is the land surface temperature (LST). Temperature at the top of the clouds is usually much lower than LST. In the MVC procedure, values of the same pixel location within the 10-day period are compared, and the higher selected in the composite product. This procedure produces an image that will not have clouds, except for the areas where clouds were present during the whole composite period (e.g., 10 days in the example). Besides temporal matters, high-resolution imagery is not always suitable for large-scale studies (regional or continental) because the sheer amount of accumulated data puts an unnecessary complexity in the analysis of data.

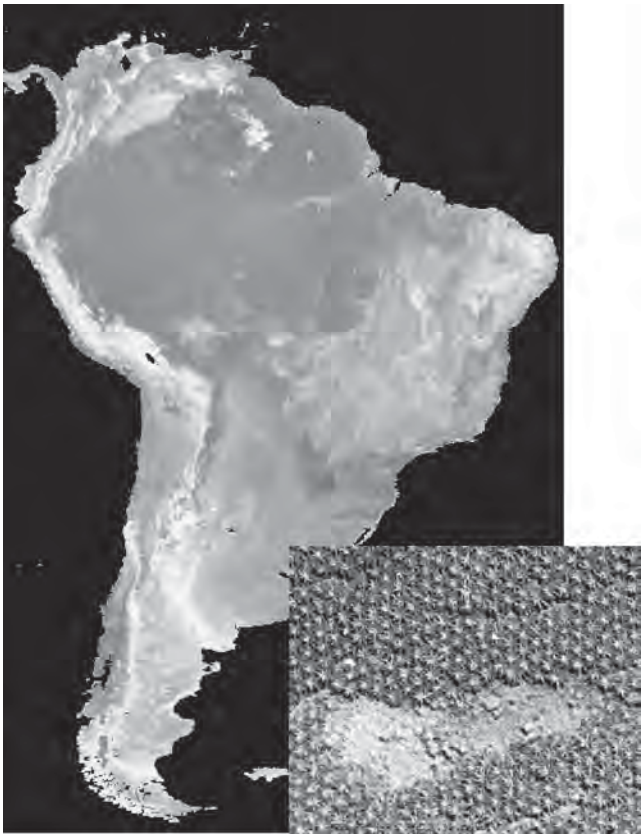


Fig. 26.1. The variety of sensors onboard earth observation satellites allow a wide range of spatial scale analysis. The bigger image represents the average of monthly values of the vegetation index (NDVI) recorded during the period 1981–2000 by the AVHRR sensor; it is a good representation of the vegetation cover heterogeneity at the continental scale (pixel size is 8 × 8 km). The smaller image was produced by the Ikonos satellite and shows the canopies of individual palms in an industrial setting southeast of Asia (pixel size is 1 m).

Remotely sensed data allow the estimation of several surrogates of meteorological variables. This is particularly important for areas where meteorological stations do not exist or do not

have the necessary density [12]. A number of algorithms using a combination of bands results in estimation of land surface and air temperature, vapor pressure deficit, rainfall, and so on [7]. These variables are relevant for the analysis and surveillance of many vector-borne disease. Since 2000, MODIS processed products were made available freely over the Internet, where a user can search the data pool by selecting a specific data group. The MODIS Land collection from the Terra mission (MOLT) is useful for vector studies. Of particular interest for epidemiological applications are LST estimates (day and night) of 1000 m spatial resolution (especially the 8-day composites) and the Normalized Difference Vegetation Index (NDVI) 16-day composites with 250 m spatial resolution. LST and NDVI products can be freely downloaded from http://lpdaac.usgs.gov/modis/mod11a2v4.asp and http://lpdaac.usgs.gov/modis/mod13q1v4.asp, respectively.

With the possibility of analyzing relatively long-time series of satellite data, it is now feasible to produce fine characterizations of areas through the extraction of time series statistics to produce environmental profiles containing all the relevant information of the series (e.g., Fourier time series analysis [30]) (see Fig. 26.2). Within a surveillance system, this environmental profiling can represent an important tool for the estimation of the most important variables of the epidemiological system under consideration.

The final utility of remotely sensed data for a surveillance system depends on finding a reliable quantitative relationship between the sensor measure and an epidemiologically meaningful variable. Remote sensors can provide estimates of a number of environmental variables, relevant for the population ecology of disease vectors, especially land cover and meteorological variables, directly or indirectly associated with the biology of vector species. Integration of environmental variables recorded by remote sensors with information collected from various sources within a spatial database is a powerful tool that can be used either to build or validate hypothesis related to the vector or the disease (see Fig. 26.3).

Early studies of vector-borne diseases using remotely sensed data were carried out in the 1970s, although the discipline started to grow with the popularization of desktop computers and massive data distribution through the Internet. During this relatively short period, the study of vector-borne diseases using remotely sensed data aimed at showing the

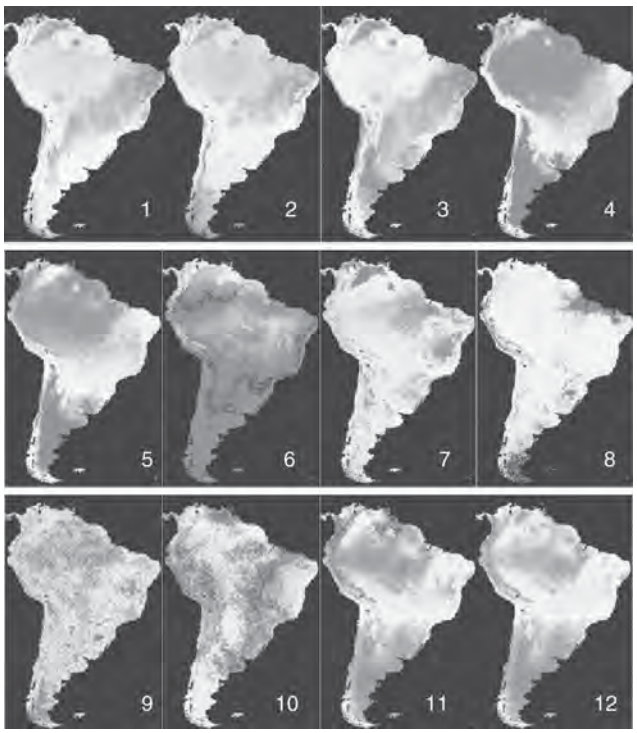


Fig. 26.2. Time series data of environmental variables recorded by remote sensors offer the possibility of estimating a number of descriptive statistics that characterize each location. As an example, this image set shows 12 statistics derived from the 1981–2000 time series of monthly images of land surface temperature (LST) recorded by the AVHRR sensor. Each image has a spatial resolution of 8 × 8 km and is presented with a blue (lower)–red (higher) color palette. 1: average, 2: minimum, 3: maximum, 4: total variance, 5 and 6: amplitude and phase of the annual cycle, 7 and 8: amplitude and phase of the biannual cycle, 9 and 10: amplitude and phase of the triannual cycle, 11: variability of the annual cycle, 12: variability of the annual, biannual, and triannual cycles. The image set was processed by and kindly provided by Dr. D.J. Rogers of the Tala Research Group, Oxford University.

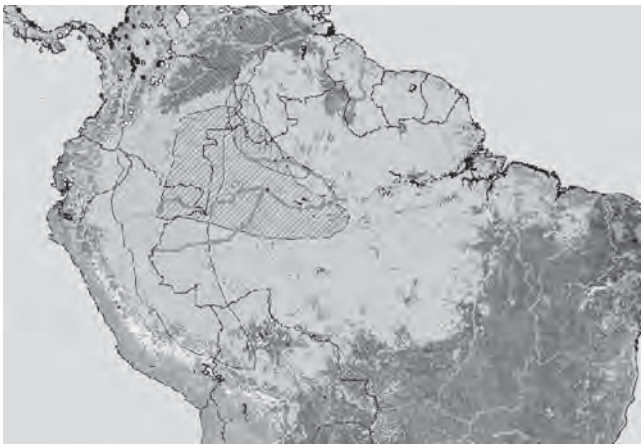


Fig. 26.3. Integration of information in a spatial database using a GIS. A GIS (described in detail in Curtis et al., this book) allows the integration of remotely sensed data with field collected data and published information. In this example, eight data layers obtained from different data sources sharing the same geographic space are integrated. The colored raster represents different land-use/land cover categories identified by NOAA–AVHRR imagery at 1 × 1 km spatial resolution (produced by the USGS; can be downloaded from http://edcsns17.cr.usgs.gov/glcc/sa_int.html); the black dots are sites where specimens *Rhodnius pallescens* were collected; white dots are sites where *Triatoma dimidiata* were collected; poligons with diagonal or vertical patterns are distribution areas of palms associated to several species of *Rhodnius*, scanned from a monography on palm species of the Americas [15] (*Attalea butyracea* (downward diagonal), *Mauritia* spp. (upward diagonal), and *Leopoldinia piassaba* (vertical)); red thick lines are proposed transects to study triatomine species of the Amazon; coastlines and political boundaries were downloaded from the Global Resource Information Database web site (http://www.grid.unep.ch/).

capacity of sensors onboard earth observation satellites to produce information on environmental variables associated with the biology of vector species. The links between the two in the form of quantitative models, together with an understanding of field epidemiology, allowed the construction of quantitative models able to describe and predict the distribution and transmission risk of vector-borne disease.

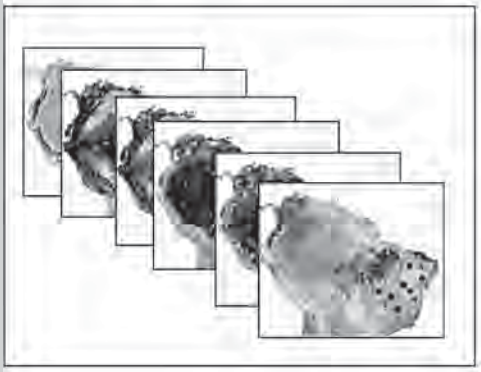
The basic procedure for the use of satellite imagery within the context of vector-borne disease epidemiology begins with the construction of maps with the known field occurrence of vectors and/or cases. Biotic and abiotic properties of sites where vectors and/or cases occur (that can be associated with vectors and/or cases) can be characterized through numerical analysis using data recorded by remote sensors. The set of

BOX 26.1. BASIC SCHEME FOR THE USE OF REMOTELY SENSED IMAGERY FOR THE STUDY OF THE GEOGRAPHIC DISTRIBUTION OF DISEASE VECTORS.

Consider a situation where specimens of two species (A and B), whose geographic distributions are not well known, were collected in sites 1-3 (species A) and 4-7 (species B) (step 1). Steps leading to prediction of the geographic distribution of the species based on remotely sensed imagery, could follow the steps below:

1. Build a collection of remotely sensed imagery.

The collection could be represented by different environmental variables, statistics derived from time series of environmental variables, or a time series of one environmental variable

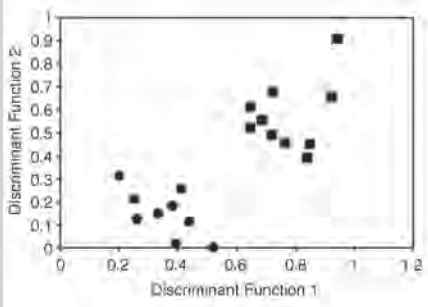


2. Extract data from the RS imagery from the interest sites

Environmental variables	sp A (sites 1 2 3)	sp B (sites 4 5 6 7)
Air Temperature		
Average	23	28
Variance	28	36
Minimum	16	14
Maximum	29	32
Land Surface Temperature		
Average	38	39
Variance	56	42
Minimum	21	26
Maximum	42	45
Vegetation Index		
Average	22	96
Variance	72	57
Minimum	12	25
Maximum	72	116
Rainfal	335	532
VPDeficit	68	52
Altitude	560	320

3. Develop a numerical model to describe species collection sites as a function of the remotely sensed data (e.g. discriminant analysis)

4. Identify variables with higher loadings for discrimination of collection sites



5. Identify areas environmentally similar to the collection sites, based on RS data and the discrimination model

environmental variables can be used as an input to build a model to describe an environmental profile associated with the occurrence of vectors and/or cases. The model can then be used to identify areas with similar environmental profiles that can be associated with the occurrence of the vector and/or transmission risk of the disease in field areas not previously visited [22] (see Box 26.1).

A number of institutions are processing, storing, and offering free access to time series of satellite imagery useful for the development of retrospective analysis, seeking the study of the relationships between existing field data and remotely sensed data. One of the longest time series is the 1981–2000 monthly MVC NDVI derived from the Advanced Very High Resolution Radiometer (AVHRR) on board National Oceanic and Atmospheric Administration (NOAA) satellite series, with 8 × 8 km spatial resolution of world coverage (NASA Goddard DAAC ftp site [21]), that has been used in a number of studies to characterize environmental profiles through the temporal changes of vegetation cover (e.g., http://glcf.umiacs.umd.edu).

The wide geographical coverage and retrospective possibility to analyze historical data of vector abundance, vector habitat, and disease epidemiology gives an enormous potential for remote sensing technology to develop models of different components of the epidemiology of vector-borne diseases. This modeling approach opens several alternatives for the construction of surveillance systems integrating field and remotely sensed data.

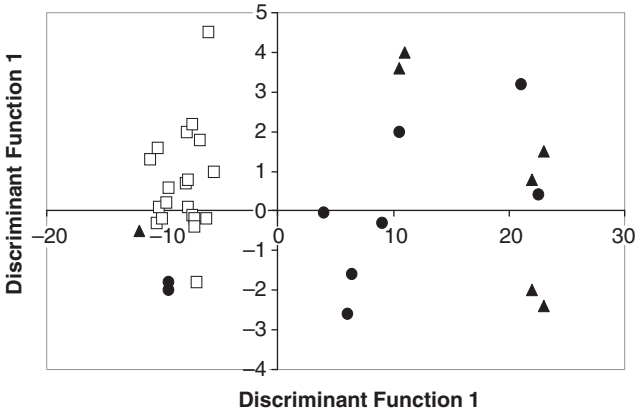


Fig. 26.4. Normalized Difference Vegetation Index (NDVI) allows the estimation of *Ochlerotatus albifasciatus* larval abundance in central Argentina. Data show a discriminant analysis of larval abundance categorized into three classes: no larvae ( ); low density (●); and high density (▼) (redrawn from Gleiser et al. [6]).

26.3 IDENTIFICATION OF VECTOR HABITATS

Remotely sensed data allow the identification of land cover, through a process known as image classification, that uses the spectral information contained in the image product. Theory of image classification is associated with the properties of energy reflection and/or emission. Different bands of an image represent the energy received by the sensors onboard the satellite platform in different wavelength ranges of the electromagnetic spectrum. Each land surface object reflects the sun's energy in a particular way that could be described by the energy values recorded in each image band. The set of energy values reflected by an object in the different bands is commonly referred to as the *spectral signature* of the object. This spectral signature can be used to identify different objects on the land surface through a process of supervised or unsupervised image classification. Supervised classification methods requires field visits to identify the objects, then use the spectral properties to produce a numerical model of the object and then use this model to identify the same kind of objects elsewhere in the image. Unsupervised methods aim at discriminating objects with different spectral properties, without reference of what the different objects are. The detail of the classification will depend on the spectral and spatial resolution of the image. Using high-resolution imagery (such as Landsat or Spot images), it is possible to discriminate different land uses, identify vegetation communities, and so on. Using low-resolution imagery, it is possible (for example) to obtain measures of vegetation activity and to identify broad land-use areas.

Remotely sensed variables have been shown to give reliable estimates of vector population parameters (i.e., mortality rate and abundance), disease transmission risk, and vector species distribution. Estimations using NOAA–AVHRR imagery (1 × 1 km spatial resolution) reported relationships between bimonthly density-independent mortality rates of *Glossina morsitans submorsitans* with LST [31], between the mean monthly mortality (female to larvae) with NDVI in *Riphicephalus appendiculatus* [27], and between weekly estimates of population abundance and NDVI in *Aedes albifasciatus* [6] (Fig. 26.4).

The estimation of the geographic distribution of several vector species using remotely sensed data has been reported in the cases of vector species of sleeping sickness [32,34,35], malaria [33,39], cutaneous and visceral leishmaniasis [24,38], blue tongue virus [26,37], tick-borne encephalitis [28], hanta virus [5], lyme disease [13], schistosomiasis and filariasis [1,19], geohelminths [1], and vectors of Chagas disease (*Triatoma infestans* and *Triatoma brasiliensis*) [4,9,10] (see Fig. 26.5).

A frequently used approach involves a description of the geographic distribution and abundance of vector species over large areas (regional or continental scales), using environmental variables (biotic or climatic) derived from remotely sensed data as ecological indicators. The link between remotely sensed data and components of the disease epidemiology may be direct, indirect, or purely statistical. Some models of infectious diseases and particularly of vector species are relatively good at describing the geographical distribution and the temporal variation of population abundance of vector species over large areas. This type of model aims at having a good predictive ability, either spatially to identify sites where the vector species is or is not present, or temporally to estimate the future abundance of the vector population, measured through the

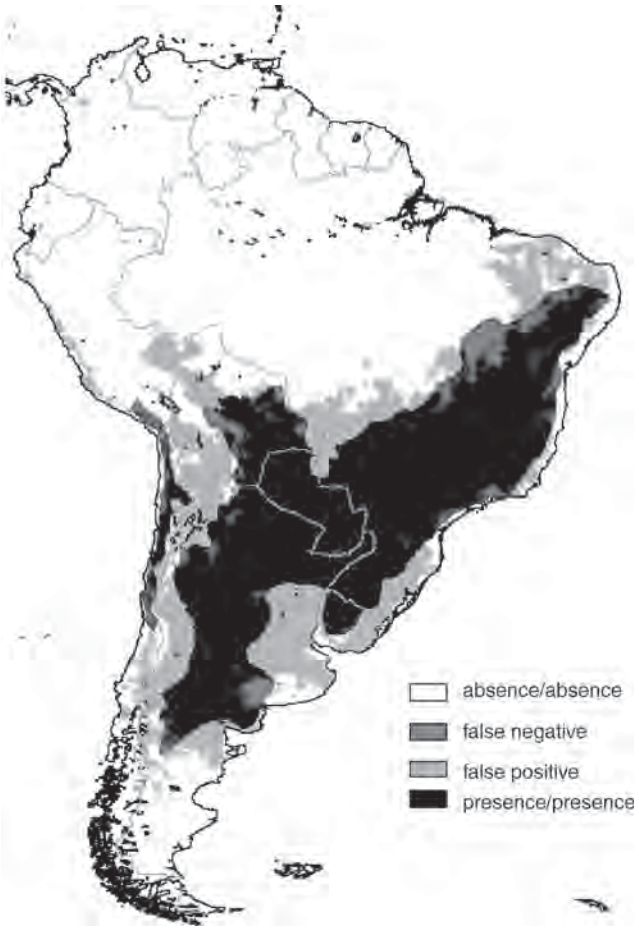


Fig. 26.5. Potential geographic distribution of *T. infestans*, estimated from a classification based on six environmental variables (four related with temperature, one with maximum middle infrared, and one with maximum of the NDVI), with the higher loadings estimated by a stepwise discriminant analysis, among 56 variables derived from a temporal Fourier analysis of the 1982–2000 time series of AVHRR images. The base map used to contrast the estimated distribution is the one published by Schofield [36] from field data. The analysis has an overall correct classification of 89% (redrawn from [9]). See color plates.

kappa index, specificity, sensitivity [29], ROC analysis [2,11], but do not necessarily intend to demonstrate cause–effect relationships between the remotely sensed data and the epidemiological variables. The most frequent numerical methods are multivariate discriminant analysis [30] and genetic algorithms applied to the ecologic niche modeling approach [23].

Meteorological variables recorded by remote sensors onboard earth observation satellites can contribute to the understanding of epidemiological indicators based on environmental variables recorded by remote sensors. A recent study by Porcasi et al. [25] showed that the heterogeneity of the distribution of houses infested by *T. infestans* in the southern area of the Gran Chaco region in the Southern Cone of South America is significantly associated to the night values of the LST extracted from 8-day composites of the 1 km spatial resolution MODIS product. The study showed that at a departmental scale (an area of 100 × 100 km), high house infestation significantly clusters at the southwest of the area. The area of high house infestation has 2–4°C higher night temperature than the area of low house infestation, although no significant difference was found comparing day temperatures between the two areas of low and high house infestation. As minimum temperature is inversely associated with density independent mortality [8], there is good evidence to use LST as a proxy indicator of potential house infestation in the area.

The environmental approach to map species distribution and abundance eventually produces a good description of distribution and abundance over large areas, sometimes over entire continents (the "statistical" approach), although it ignores the influence of intrinsic factors of the vector population that regulates its abundance. From this perspective, the resulting description (if appropriately validated) can be an important tool for the decision process of regional health authorities, but at the same time has the weakness of not identifying cause–effect relationships (that are normally studied to find mechanistic explanations through the "biological" approach) [30].

The statistical and biological approaches have their own strengths and weaknesses. Better understanding of the problems under consideration will come when one complements the other. In this stage of the technology and knowledge about the application of remotely sensed data to studies of vector-borne diseases, the next step is the incorporation of statistical and biological knowledge within surveillance programs using geographic information systems that can be progressively improved by expansion of the knowledge base and subjected to dynamic validation.

26.4 MONITORING ENVIRONMENTAL CHANGES FOR DISEASE SURVEILLANCE

Several disease outbreaks are associated with changes in land cover, especially deforestation. With the ability to track land use over time, remotely sensed data can be used to monitor land cover and land-use change. Land cover estimated using remotely sensed data has predictive power to describe the distribution of cutaneous leishmaniasis in Colombia [18] and outbreak occurrence has been associated with deforestation edges, but there is a need for ground studies to analyze the mechanism and quantify the relationships.

26.4.1 The Case of Chagas Disease in the Amazon

The Amazon region is large, biologically complex, and subject to rapid land-use change, with approximately 2 million ha per year being deforested. Satellite data show that over half the deforestation involves small clearings of 15–100 ha, representing small farmers who have immigrated from other regions. The 1999 census of Brazil indicates there are now 12.1 million

inhabitants of the Amazon region of Brazil, and yet the infrastructure of the region remains extremely underdeveloped and access to the majority of sites is very difficult [3,17].

Elsewhere in Latin America, most cases of Chagas disease transmission depend on domestic vector populations that live in the cracks and crevices of roof and walls and emerge at night to feed on the sleeping occupants. This is not the case in the Amazon region, where a variety of other transmission patterns is being identified. *R. brethesi* flies from piassaba palms (*Leopoldinia piassaba*) to attack palm fibre collectors; *R. pictipes* flies into houses, apparently attracted by lights, and is frequently triturated in the machines used to make açai (a palm species) juice – almost 50% of the acute cases of Chagas disease so far recorded in the Amazon region appear to have been microepidemics amongst families drinking açai juice contaminated by triturated *Rhodnius*; *Panstrongylus geniculatus*, once thought to be extremely difficult to rear in artificial habitats, is now found colonizing peridomestic habitats in several areas; in Marajó it feeds on domestic pigs, but when the pigs are set out to feed, it switches to humans causing severe cutaneous lesions by its bite [40]. *R. robustus*, once thought to be species of savanna palm trees, is now shown to involve at least four different genotypes in the Amazon region, of which the behavior of only one is known. The other species of Triatominae recorded from the Amazon forest region are of virtually unknown habit.

The existing surveillance and control procedure for Chagas disease in Latin America is based on the presence or absence of domestic Triatominae, detected by householder reports or active house-to-house searching. Such an approach is impractical for the Amazon region, because of the complexities of the different transmission cycles and the difficulties of access to most of the Amazonian communities. A new approach is required, with a methodology able to consider the land change and land cover changes, the ongoing antropization process of the region, and the large size of the area.

We do not yet know the factors that trigger domestic invasion by Triatominae, although for some species this seems to depend largely on land-use changes causing mortality of reservoir hosts, leading to starvation of the bug populations that can trigger adult flight.

The loss of biodiversity leads to an increase of Chagas disease transmission in recently colonized areas in the Amazonian region. This is because forest clearing to establish pastures, usually leaves isolated palm trees for shading cattle. These isolated palms represent island refuges for several vertebrate species, which in turn are hosts of *Rhodnius* species. So, a palm tree landscape leads to a concentration of *Rhodnius* bugs, that eventually fly into newly established human houses.

This complex, large, and challenging scenario is a good opportunity for the development of a surveillance system using remotely sensed data to estimate the geographic distribution of palm species to which *Rhodnius* species are associated, and to detect and predict areas subjected to land cover change, as part of the process of the Amazonian colonization and its eventual association with the occurrence of Chagas disease cases. This remote sensing component, together with field surveillance of cases, will conform an integral surveillance system that will eventually help in the consolidation of this regional initiative for the control of the American trypanosomiasis.

26.5 EARLY WARNING SYSTEMS FOR VECTOR-BORNE DISEASE OUTBREAKS

The ability of passive remote sensors to produce a continuous stream of meteorological and land cover data with complete spatial coverage offers an ideal opportunity to develop early warning systems (EWS) of infectious disease outbreaks [20]. A number of studies show that changes in environmental variables (mainly climatic and land cover variables) are associated with vector abundance and/or the number of disease cases in a particular region. In each case, knowledge of the vector biology, pathogen transmission dynamics, and their relationships with environmental variables is crucial for the construction of predictive models of infectious disease outbreaks. Historical studies show the usefulness of long-term datasets to build models of present and future disease pattern, suggesting ways of using historical data to construct EWS based on overall association between disease incidence and the environment, without mandatory use of complete knowledge of the relationships between different components of the disease transmission cycle and the effects of the environmental variables. This is a convenient way of using existing data, either about the disease or the environmental information of remotely sensed databases. However, more robust predictions could be based on prospective studies aiming at validation of the disease components.

EWS are extremely appealing, as they would allow advance preparation of the health system to confront future outbreaks. However, it is only recently that their use is being studied at the research level and their effectiveness needs the validation of ongoing infectious disease control programs. EWS are of little use if the capacity to respond is not present, and experience with famine EWS in the 1990s showed that their effectiveness depended less on the accuracy of warning than on political factors.

Remote sensing and geographic information systems technology has been sometimes presented as the final solution for the surveillance of several vector-borne diseases. Although remote sensing has much to offer, it has some way to go before it becomes a tool integrated into national programs for vector control [42]. There is an urgent need for local field studies to produce reliable representations of epidemiological parameters through remotely sensed data, of the involvement of health personnel into model developments and construction of surveillance systems including satellite imagery, and evaluation of the practical applications of the developed model based on remotely sensed data to specific disease control programs.

General perception suggests that the integration of RS and surveillance data in a GIS, together with ancillary data on

human population demography, is the basis for development of effective EWS. There is particular interest in methods of spatial epidemiology that allow for the detection of disease clustering in space and/or time [22] that can be used to stratify disease risk. Analysis of the association between such disease clusters and environmental variables measured by remote sensors could represent a valuable tool kit for the allocation of resources for the prevention of disease occurrence in a cost-effective fashion.

ACKNOWLEDGMENT

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CHAPTER 27

Archaeological Epidemiology of Infectious Diseases: Fossil DNA

Felipe Guhl[1] and Arthur Aufderheide[2]

[1]*Faculty of Sciences, Universidad de los Andes, Bogotá, Colombia*
[2]*Department of Pathology, University of Minnesota, Duluth, USA*

27.1 INTRODUCTION

Living creatures arose nearly 3500 million years ago (mya) and life on Earth unfolded in a slow but spectacular panorama of events. Microorganisms have dominated the planet for much of its history and adapted to terrestrial environments prior to the appearance of modern humans. Since the beginning of the evolutionary process, living organisms adapted successfully to many different environments including the living environment itself.

Such a long interval accommodated the adaptation of various infectious agents to their new hosts. The ancestors of present-day man (*Homo sapiens sapiens*) appeared in East Africa some 3.5 mya (*Australopithecus*) and then migrated to Europe, Asia, and later to the Americas. The first human migrations were accompanied by the three human plasmodia (*Plasmodium falciparum, P. vivax*, and *P. malariae*) imported from Africa into the Mediterranean region, but it was the Neolithic revolution which created actual malarial foci [33]. Cultural developments, such as agricultural and permanent or semipermanent settlement patterns, the passage from nomadic to sedentary life (*c.* 8000 B.C.) all created ideal conditions for the spread of other infectious diseases such as tuberculosis and syphilis (Box 27.1 and 27.2).

A recent literature review [17] reports that 133 cases of prehistoric tuberculosis have been studied by different authors and, just as is true today, transmission of the infection and establishment of the disease were favored by cultural and life style changes such as sedentism, crowding, under-nutrition, use of dark and isolated habitats, and the frequency of interpersonal contacts.

Hosts offered not only permanent availability of food and protection but also an effective mechanism of dispersion to every organism which knew how to exploit it. Spontaneous or forced human migrations including domestic animals led to enormous mixtures of populations on the different continents, favoring the spread of numerous infectious agents throughout the evolutionary process (see Chapter 19).

On the other hand, epidemiological patterns of many of the vector-borne diseases were initially restricted to particular cycles in specific environments where the primitive transmission of microorganisms took place without human intervention.

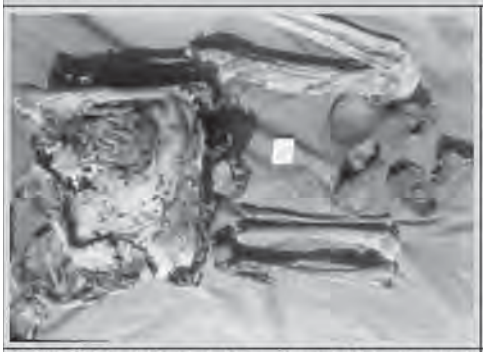
As humans came into contact with the natural foci of infection, and caused different degrees of ecological transformation, they became infected with many different microorganisms, such as plasmodia, trypanosomes, leishmania, and yellow fever virus. Human fossils have taught us that our species has been plagued by infectious diseases throughout the prehistoric, preliterate period.

The importance of infectious diseases is expressed not only in their current or past impact on human populations' mortality or fertility rates or their economic or medical repercussions. Infectious agents provide useful data, especially when identified from the mummified remains of humans and

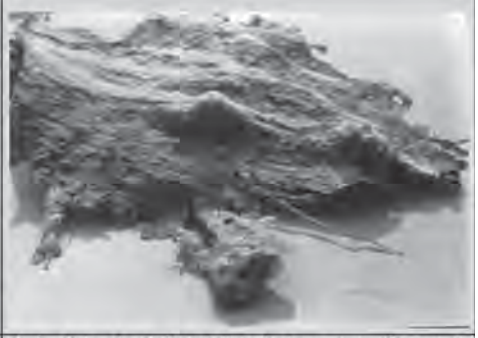
Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

Box 27.1. TUBERCULOSIS

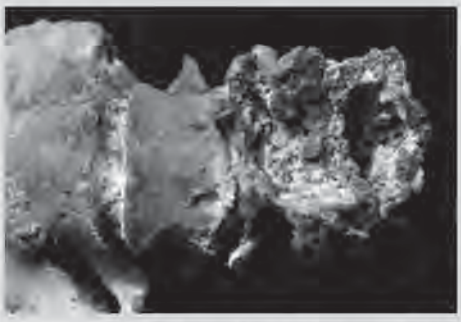
Tuberculosis is the most common major infectious disease today, infecting two billion people or one-third of the world's population, with nine million new cases of active disease annually, resulting in two million deaths, mostly in developing countries. Is an infection with the bacterium *Mycobacterium tuberculosis*, which most commonly affects the lungs (pulmonary TB) but can also affect the central nervous system (meningitis), lymphatic system, circulatory system (miliary TB), genitourinary system, bones and joints.



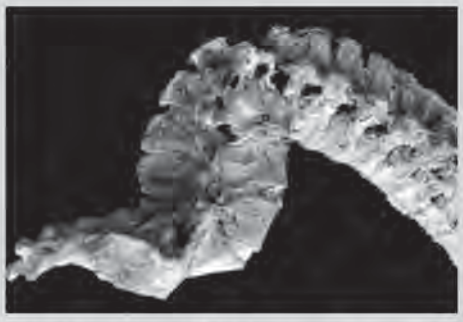
A. This 35 year-old female body was fragmented by ancient tomb looters. The thorax, however, was intact. Within it were the lungs and mediastinal tissue illustrated in Fig.6. Chiribaya-related culture from a coastal desert site, southern Peru, c.AD 1000. Photo by author A C A.



B. A subpleural, calcified shell in the lower center (foreground) was found in the right lung. The partly calcified, nodular elevations in the mediastinal tissues are lymph nodes. From which aDNA of the tuberculosis complex bacteria was isolated and sequenced. Same individual as in A. Photo by author A C A.



C. Demonstrates three lumbar vertebrae. Two are intact. The third reveals almost complete destruction of the vertebral body. Note the absence of any new bone formation in response.



D. The function of the vertebral body is to support the upper half of the human body's weight. Tuberculosis often infects and destroys the vertebral body. Since the vertebral arch often remains intact when the infected vertebral body collapses and the upper half of the human body is bent forwards (angular kyphosis or gibbus). This generates the humpbacked posture described by Sir Percivall Pott in 1799 and demonstrated in this photo.

animals found in a particular archaeological excavation site. Precise information can be obtained about their evolutionary processes, phylogeny, geographical distribution, and their hosts and reservoirs in the case of parasites.

A clear idea regarding the contact which different civilizations or population groups could have had amongst themselves, their migratory movements and possible extinction can also be deduced when medical effects are integrated with archaeological data.

Evidence must be correctly documented and interpreted. Other sciences such as geology, osteopathology, chemistry, anthropology, and molecular biology often can provide valuable additional information. The latter science particularly has gained enormous ground during the last few years. Given its high level of resolution and sensitivity, it has become a very valuable ally for studies and analysis in the area of paleomicrobiology. The techniques briefly described in this chapter range from the simple observation of coprolites, their shape (scatology), and content to the application of modern laboratory procedures which has led to testing hypotheses regarding parasite evolution and host–parasite relationships.

27.2 TECHNIQUES AND PROCEDURES FOR DETECTING INFECTIOUS AGENTS IN ARCHAEOLOGY

Sources of data from which information can be obtained.

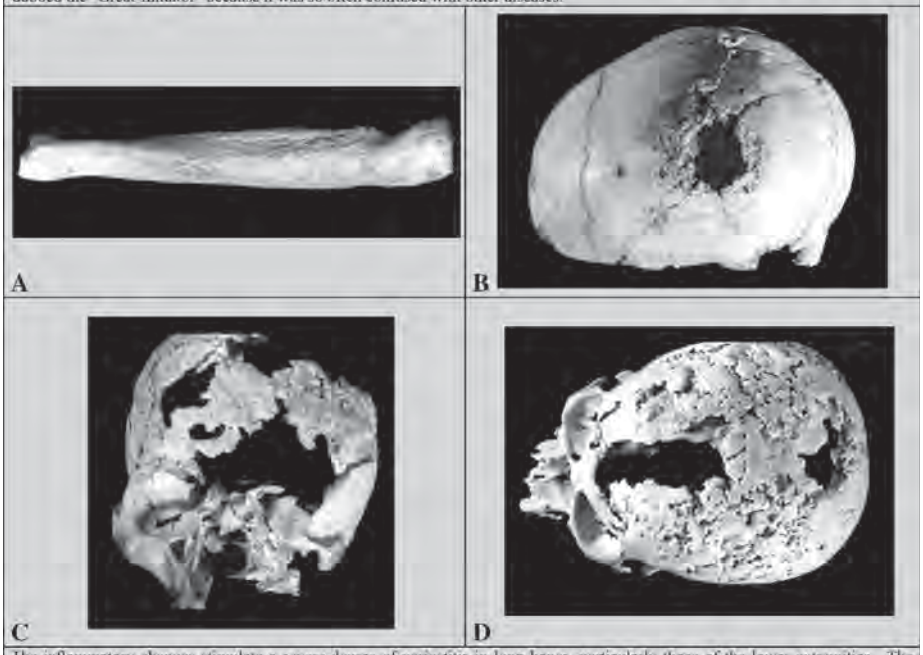
27.2.1 Mummies

In this chapter the term "mummy" is used for ancient corpses in which sufficient soft tissue was preserved to resemble human bodies [3].

Mummified human and animal bodies have been found in many places around the world. In only a relatively few have infectious agents been found. However, some pioneering work has provided excellent protocols and models for paleoparasitological evaluation such as the interesting report regarding the finding of *Trichiurus trichiura* eggs in the intestines of an Inca victim destined for being sacrificed [35]. Mummies represent the most important potential source of information for human paleoparasitology having been able to shed light on some aspects regarding different origins. Human migration routes to the Americas are a good example. Some parasites are endemic

Box 27.2. TREPONEMATOSIS

is a sexually transmitted disease (STD) that is caused by a spyrochaete bacterium, *Treponema pallidum*. Syphilis has many alternate names, such as: Miss Siff, the Pox, and has been given many national attributions, *e.g.* the French disease or the English disease. The signs and symptoms of syphilis are myriad; before the advent of serological testing, diagnosis was more difficult and the disease was dubbed the "Great-Imitator" because it was so often confused with other diseases.



The inflammatory changes stimulate a severe degree of periostitis in long bones, particularly those of the lower extremities. The consequence is a substantial thickening of the cortex, the surface of which is remarkably irregular as seen in the tibia in A. In cranial bones osteal infarcts (gummas) develop commonly syphilitic arteritis. These may involve only the outer table, only the inner or both. The necrotic material may liquefy and be resorbed, with a resulting collapse of the table bone, producing a surface depression. Larger infarcts may involve both outer and inner table as well as the trabecular bone between the two tables. Resorption of such large gummas causes a complete defect in the affected cranial vault bones. At the periphery of such lesions the related inflammation stimulates new bone formation, generating a peripheral rim of dense, sclerotic, bone surrounding the defect. All of these features can be identified in the accompanying images of cranial syphilis (**B, C & D**).

Fig. 27.1. Extremely well preserved human mummified body belonging to the Colombian high plains Region (450 years old) (photo by author F.G.)

to America and others accompanied humans since their origin in Africa. Parasites can thus be considered as hominid markers (i.e., elements which may provide clues regarding the evolution of this group of primates).

Infectious agents' evolutionary history can also be reconstructed at the same time. An exceptional and recent study [2] reported the 9000-year-old record of Chagas disease (Box 27.3) Tissue specimens from 283 principally spontaneously (naturally) desiccated human mummies from coastal and low valley sites in northern Chile and southern Peru were tested with a DNA probe directed at a kinetoplast DNA segment of *Trypanosoma cruzi*. The time interval spanned by the eleven major cultural groups represented in the sample ranged from 9000 years B.P. to approximately the time of the Spanish conquest, in 450 B.P. Forty-one percent of the tissue extracts were amplified by polymerase chain reaction (PCR) and hybridized with the probe. Prevalence patterns demonstrated no statistically significant differences among the individual cultural groups. These results suggest that the sylvatic cycle of *T. cruzi* was probably well established at the time that the earliest humans, members of the Chinchorro culture, first peopled this region of the Andean coast and inadvertently joined the many other mammal species acting as hosts for this parasite [19].

Box 27.3. CHAGAS DISEASE

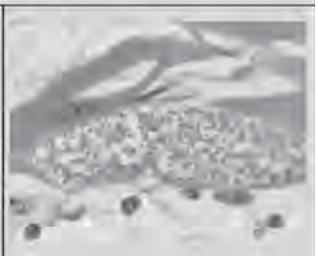
As humans came into contact with the natural foci of infection might then have become infected as a single addition to the already extensive host range of *Trypanosoma cruzi* that includes other primates. Thus began a process of adaptation and domiciliation to human habitations through which the vector had direct access to abundant blood as well as protection from climatic changes and predators.

Photo by author F.G.

***Trypanosoma cruzi* (Giemsa x 950)**
Also known as Chagas disease, American trypanosomiasis is zoonotic infection caused by a protozoan parasite, *T. cruzi*. The disease is found in Central and South America. The causative agent occurs in blood films characteristically as short 'C' or 'S'-shaped trypomastigotes with a prominent kinetoplast.

Photo by author F.G.

Rhodnius prolixus
R. prolixus is one the most widely distributed vector species in the Andean countries and central America. The insect transmit *T. cruzi* while feeding, not by inoculation but by faecal contamination. The insects normally hide in the walls during the day time and emerge after dark to feed on the sleeping inhabitants.



Courtesy Dr. Gerzain Rodriguez, Laboratorio de patologia, INS Bogota Colombia

Heart lesion (haemotoxylin x 350)
Thin section of heart muscle (haemotoxylin & eosin stain) showing amastigote stage of *Trypanosoma cruzi*. Amastigotes multiply, destroying adjoining tissue, and form pseudocysts. Individual who survive the acute stage are often left with chronic and progressive neuronal and smooth muscle lesions in the heart and gastrointestinal tract.

More information about American trypanosomiasis is in Chapter 25.

The use of mummies as well as coprolites from around the world has enabled researchers from different disciplines being able to predict ancestral ways of life, customs, and possible causes of death. These causes vary considerably, including trauma, hereditary diseases, environmental conditions, and infectious diseases.

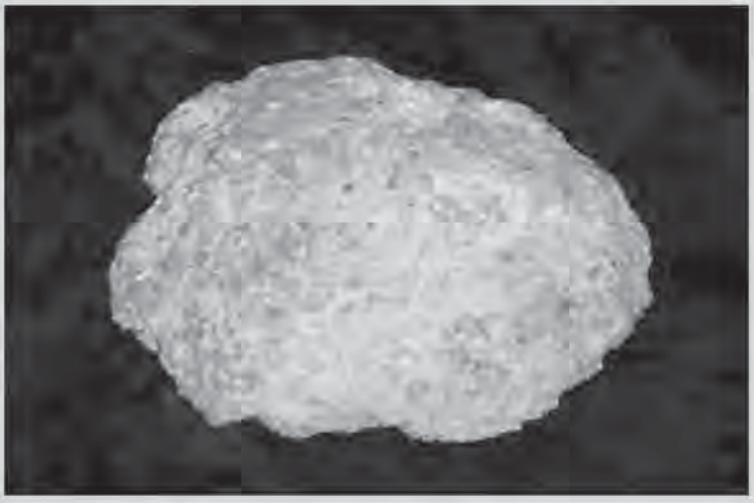
27.2.2 Coprolites

Samples of desiccated feces perhaps provide the most immediate source of information. Such data obtained from coprolites are basically restricted to helminthes, excluding most other infectious agents (Box 27.4).

The study of feces from terrestrial mammals brings out biological and ecological data such as the presence of a particular species, their diet, behavior, territory, parasitic fauna,

Box 27.4. COPROLITE

is the fossilized excrement of animals. Under the right conditions animal faeces have been fossilized (preserved) are called coprolites. Animal faeces are mainly composed of soft faecal matter and sometimes have bone fragments, and are usually rarer than skeletal type fossils.



http://www.fossilguru.com/coprolitefossil.asp?sm

Coprolites - dung mostly recognized by their shapes they can vary considerably in size and shapes colors and forms, coprolites can be flat pancake like shape spherical spiral, a big blob, some you can even see the pinch marks, and others may contain bone fragments, some may be white, brown, black, and multiple colors. Coprolites can come from sharks, gators, turtles, and many other types of animals. Photograph shows a fossilized dinosaur dung, 65 mya old.

Fig. 27.2. This spontaneously mummified body of a 14-year-old male was excavated from a Tiwanaku-related, coastal desert site in northern Chile, dated to *c.* A.D. 1000. His enlarged, exudate-covered heart is evident in Figure 27.4 (photo by author A.C.A.).

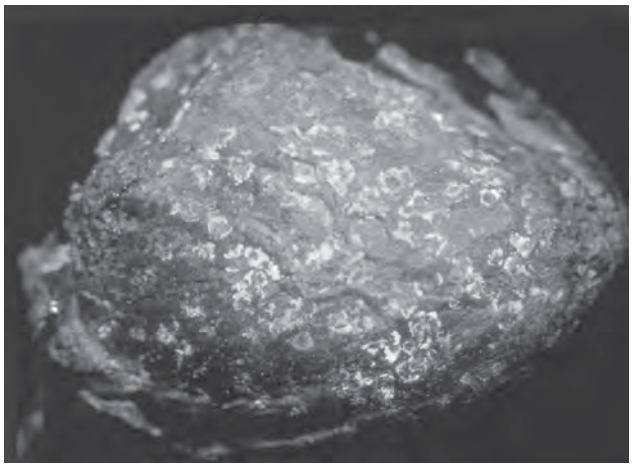


Fig. 27.3. This heart was present in the thorax of the body in this figure. The light areas represent exudates. The aDNA unique to *Trypanosoma cruzi*, the parasite for Chagas disease (American trypanosomiasis), was isolated from a soft tissue (colon) sample (photo by author A.C.A.). See color plates.

and peridomestic use [5]. Morphometric analysis also permits the primary identification of the taxonomic group origin to support the best choice of subsequent analysis [9].

Coprolites are frequently found in mummified remains and can be rehydrated and reconstructed by the simple method of submerging them in a 0.5% trisodium phosphate solution. Once the reconstituted material has been obtained, it can be concentrated by using centrifuging formol-ether or flotation methods to enable better detection of possible parasite material present. Helminth eggs, larval fragments, or cysts are some of the elements which can be recovered and identified by rehydrating coprolites.

Molecular paleoparasitological diagnostic approach was recently developed for *Enterobius vermicularis* [27]. The authors extracted ancient DNA (aDNA) from 27 coprolites from archaeological sites in Chile and the United States, suggesting that paleoparasitological identification by aDNA analysis could complement histological diagnosis.

Almost all known human specific helminthes have been found in ancient feces. Carvalho Goncalves et al. [8] have published an extensive review of the parasitological helminth and intestinal protozoa. They have also reported new findings and suggested that *Ascaris lumbricoides* and *Trichuris trichura* found in human remains in the New World were not introduced into the Americas by land via Beringia, arguing that the parasites could not survive the cold climate of the region, in contrast with the hypobiosis hypothesis which is dealt with later on (see Box 27.5.)

27.2.3 Histological Methods

Various mummified tissues can also be carefully rehydrated and sectioned for staining and light microscope observation. Since mummified tissues usually lack intact epithelium, stains selected for connective and muscular tissue, such as Masson's trichrome stain, are more informative for mummified tissue fixed on slides. The first reported histological study under the microscope of any human mummified tissue was probably made by the Viennese physician Johan Czermak [12], when examining an Egyptian mummy skin.

By 1938 Shaw had described the histological appearance of many different viscera found in a canopic jar in the tomb of an Egyptian mummy, noting evidence of anthracosis, pulmonary emphysema, and pulmonary adhesions [44]. More extensive tissue sampling and careful gross evaluations subsequently led to a broad range of diagnoses in mummified human remains. Readers interested in more detailed reviews of this field may wish to consult Aufderheide [3].

Other preparations merit being observed by electron microscope [31]. Studied the intestinal lining of the 10,000-year-old Yuribey mammoth mummy and found spherical and linear structures suggestive of virus structures. The presence of intracellular parasites has been identified in infected samples. Fornaciari et al. [14] have reported the presence of amastigote forms of *T. cruzi* in a section of dilated esophagus from a Peruvian Inca mummy.

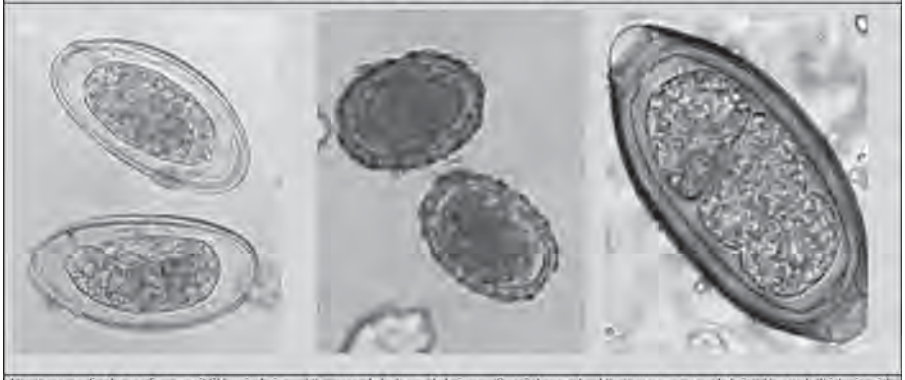
27.2.4 Immunological Methods

Recovery of antibodies from mummified tissues permits identification of infections ancient settlers suffered. Affinity chromatography showed high immunoglobulin affinity (immunoglobulin G: IgG coupled to sepharose 4B). Hydration of the most vascular tissues such as the heart, liver, and spleen in a trisodium phosphate solution, it became possible to purify IgG antibodies in sufficient concentration for carrying out diagnostic examinations using conventional tests such as indirect immunofluorescence antibody test (IFAT) or ELISA. Immunoglubulins isolated by this method can shed light on determining the etiological agent of fungal, viral, and bacterial infection [18].

Monoclonal antibodies constitute a valuable tool offering very high specificity in conventional diagnostic assays such as ELISA, IFAT, or radio-immuno assay (RIA). Monoclonal antibodies can be produced in the laboratory and can be directed against virtually any type of antigen. This reagent tool can identify parasite antigens in tissues by using the immunoperoxidase (IP) or IFAT techniques and also in paleofeces.

Box 27.5. HELMINTHES

The identification of parasites in ancient human faeces is compromised by differential preservation of identifiable parasite structures. Helminth eggs are some of the elements which can be recovered and identified by rehydrating coprolites. See color plates.



http://www.medic.ulpgc.es/fper/parasit/EVermicularis.html

Enterobius vermicularis

Enterobiasis is a worldwide in distribution and is a group infection, more common in children than adults. It occurs in family groups, especially under crowded conditions. When one infection is found it is likely there are other also. The adult worm lives in the upper part of the colon where minute ulceration may develop. At time hemorrhages occur and secondary infection causes ulcers and submucosas abscesses.

http://www.dpd.cdc.gov/dpdx/images/ParasiteImages/A-F/Ascariasis/ascaris_egg1.jpg

Ascaris lumbricoides

(Roundworm) is one of the most common and most widespread human infections. Possibly one in four of the world's population is infected. Pathology may be caused by migrating larvae or adults. Migrating larvae cause symptoms from their actual physical presence and the immune reactions they elicit. Damage to the lungs occurs during the migration of larvae on their way to the intestine. *Ascaris* liver abscess is caused by female worms migrating up the common bile duct into the liver where they die, releasing eggs.

http://www.paru.cas.cz/helmint/Nematoda/Trichuris_trichura01.jpg

Trichuris trichura

Trichuris trichura is primarily a human infection but ***Trichuris suis*** of pigs in indistinguishable form the human species and cant infect man. There is an increased incidence of *Trichuris* infection in people handling pigs and is common in areas of high rainfall, dense shade and poor sanitation and contaminated soil. The worms cause hemorrhages, mucopurulent stools and symptoms of dysentery with rectal prolapse.

Monoclonal antibodies are obtained by fusing myeloma cells and sensitized lymphocytes. The resulting hybrids conserve the property of myeloma's rapid cellular multiplication and the characteristics of synthesis and lymphocyte antibody secretion. They can be cloned and kept indefinitely in cellular culture media or frozen. An enormous range of monoclonal antibodies can be found today covering a wide diagnostic spectrum.

A recent report demonstrated the detection of *Entamoeba histolytica* in prehistoric human fecal remains using a commercially available ELISA kit employing monoclonal antibody-peroxidase-conjugate specific for *E. histolytica* adhesion [7] (Box 27.6). The test detected 20 positive samples from archaeological sites in Argentina, United States, France, Belgium, and Switzerland, from a total of ninety specimens dated 5300 years B.P.

27.2.5 Fossil DNA

Recovering and analyzing aDNA from paleontological, archaeological specimens, examples of plants and animals conserved in museums as well as forensic specimens, represent a potential target for genetic analysis.

The term fossil DNA or aDNA refers to any bulk or trace of DNA from a dead organism or parts of it, as well as extracorporeally encountered DNA of a living organism. Any DNA which has undergone autolytic or diagenetic processes or any kind of fixation is therefore considered to be "aDNA" [24]. Detecting DNA in ancient remains (ranging in age from less than 100 years to tens of millions of years) has led to fascinating new research areas having many implications, opening up the possibility of studying evolution at the molecular level over a mainly unlimited timescale [24]. Studying fossil DNA is a young field and is emerging as a valuable tool for reconstructing the past and particularly the origin of human infectious diseases. When targeted *in vitro* DNA amplification by PCR was invented in the mid-1980s, it revolutionized molecular biology and opened up new fields for applying genetic analysis. Diverse disciplines such as archaeology, forensic science, paleoparasitology, paleoepidemiology, and agriculture have all been influenced by aDNA analysis. Diverse applications emerging from research into aDNA include

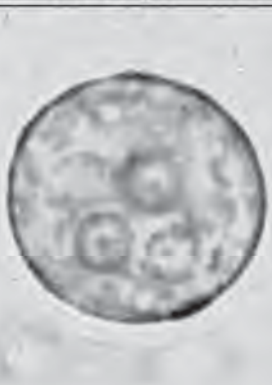
1. aDNA recovered from mummified human and animal tissues
2. aDNA recovered from coprolites
3. Recovery of aDNA from organisms trapped in amber
4. aDNA recovered from museum specimens
5. aDNA isolated from plant seeds or compression fossils
6. aDNA isolated from ancient cesspits' organic sediment

27.2.6 Molecular Biology Methods

In addition to immunological methods, molecular techniques have been used. Their review by Sallares and Gomzi [42] is not encouraging for the present state of technology. Taylor et al. [49] has recovered malarial aDNA from a rib of a 60-year-old burial. Sallares and Gomzi [42] did recover malarial aDNA from a Roman period skeleton but nonmalarial sequences were also amplified by the same primers that targeted conserved

Box 27.6. ENTAMOEBA HISTOLYTICA

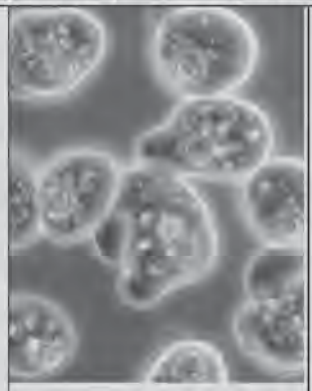
Protozoan detection in coprolites is not a frequent finding in paleoparasitology. Cysts quickly decay in the environmental conditions of archeological sites. However protozoan antigens remaindetectable for longer periods of time. See color plates.



http://www.medlib.med.utah.edu/parasitology/AllSmallJpg/EhistCystIodine.jpg

***Entamoeba histolytica* cysts**

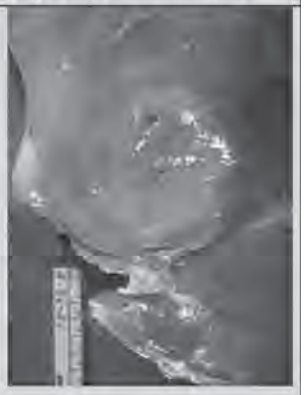
Entamoeba histolytica has a worldwide distribution and is endemic in most countries with low socio economic conditions infection occurs via the faecal-oral route. food and drink becoming contaminated through exposure to man faeces. *E. histolytica* cysts are evacuated in the stool of infected individuals and discharged into the environment. Cysts remain viable and infective for several days in faeces and water.



http://www.biosci.ohio-state.edu/~parasite/histolytica_phase.html

Trophozoites *Entamoeba histolytica* (phase contrast x 1200)

Moving trophozoites containing ingested erythrocytes may be found in a freshly passed specimen. They are clear evidence of infection.



http://pathmicro.med.sc.edu/parasitology/amebic-ab-1.jpg

Amoebic liver abscess

Gross pathology of liver containing amebic abscess. *E. histolytica* has the capacity to destroy almost all tissues of the human body. The intestinal mucosa and the liver are the most commonly affected.

ribosomal RNA genes. More research is required to carry this approach to a satisfactory level of certainty.

Contamination represents PCR's main drawback. While PCR sensitivity makes it invaluable when analyzing scarce remains, it renders it vulnerable at the same time, to the constant threat of contamination by foreign DNA both outside and within the laboratory, especially from less degraded DNA originating from modern humans. Modern DNA is a far better template for PCR amplification than aDNA, and serious precautions have therefore to be taken for minimizing the possibility of contamination and maximizing the validity of its specific results.

Some scientific reports have suggested that contaminating DNA can be amplified from modern organisms present in the tissue specimens. It has also been argued that it is theoretically implausible for DNA's chemical structure to survive for millions of years without becoming completely degraded [30]. There seems to be strong evidence, however, that a number of these sequences have actually been obtained from fossilized DNA, because phylogenetic reconstructions derived from such data agree well with the fossils' known paleontological identity [45]. Nevertheless, such examples from specimens of extreme age will probably acquire more general credibility when the results can be confirmed by independent laboratories.

The morphology of a particular microorganism can lead to misinterpretation. Hence, the use of molecular biology methods is advisable.

Multicellular organisms' fragile structures can be easily destroyed over a period of time, thus hampering the work of identification. It is difficult to detect ancient unicellular parasites by conventional, morphologic diagnostic methods. Molecular biology has become a powerful tool in genetic study allowing aDNA from mummified human bodies and also those of animals to be analyzed. These techniques also provide a very valuable source of information from the parasitological point of view. Organisms which parasitized ancient humans can often be detected, meaning that correlations can thus be established between microorganisms and their hosts (e.g., migration routes and interrelationships between different population groups can thus be deduced).

aDNA is present in a fragmented form, usually of segments less than 200 nucleotides in length and can be amplified today from *in situ* histological sections, revealing the amplification by using radioactive or enzymatic material as a "label."

An eukaryotic genome presents numerous copies of 5S and rRNA genes; these genes form part of repeat DNA segments. The hundreds or thousands of copies of the main rRNA gene are known as ribosomal DNA (rDNA) and are found grouped in the nucleolus region of one or more of the genome's chromosomes. It has been shown that numerous transcripts are synthesized at the same time from a single rRNA gene and that all rRNA genes organized in tandem simultaneously participate in transcription.

A determined species may present intergenic spacer regions between ribosomal genes with similar (not identical) sequences and hence indicating the grade of homology between closely related organisms. This could imply that there is no selective pressure for maintaining a determined spacer

sequence presenting numerous variations between one species and another. There are sequences within ribosomal genes which have evolved relatively slowly and have been exposed to strong selection pressures, meaning that they have stayed constant ("conserved") in individuals. On the contrary, these genes' spacer sequences have evolved rapidly, presenting great variability within species from the same genus or within populations. These facts allow phylogenetic relationships to be found between individuals, bearing in mind that those sequences remaining constant will allow distantly related individuals to be studied while greatly varying spacer sequences will be of interest for studying closely related individuals.

Nucleic acids are very stable molecules. A Chinese team carried out the first successful aDNA and aRNA extraction from the rib cartilage from the Old Lady of Mawangtui, a corpse preserved for almost 2000 years [26]. The first aDNA was amplified in 1984 from a museum specimen of an animal called quagga, a relatively recently extinct horse-like creature (Higuchi et al. [25]). A mitochondrial DNA fragment from this specimen was PCR-amplified and sequenced.

Molecular cloning of aDNA from 2400-year-old Egyptian mummies was achieved [34], and DNA fragments have been amplified from a 5500-year-old human bone [22].

These discoveries were soon followed by *T. cruzi* DNA isolated in 4000-year-old mummified human tissue from northern Chile [20,21], and recently, the aforementioned 9000-year record of Chagas' disease [2]. DNA fragments were being amplified from 18 million year-old *Magnolia* leaves [16], and 25–30 million year-old fossil termites in amber [13]. However, to date the identification of aDNA in multimillion year-old samples has not yet been confirmed in other laboratories.

27.2.7 Paleoparasitology

This is a relatively new discipline based on the concept of parasitism as a source of variation and evolution. This science benefits from knowledge generated by other disciplines such as history, anthropology, medicine, palaeontology, palynology (the study of pollen), geography, molecular biology, and genetics in helping to reconstruct the history of life on our planet involving all these disciplines.

Another important branch of science helping to shed light on these topics is epizootiology which studies disease patterns in animals. This allows the distribution of species to be correlated with pathological impact, the abundance of species, and possible migrations.

Readers interested in more details may wish to consult Chapter 2.

27.2.8 Paleopharmacology and Ethnography

It is most probable that ancient human populations all over the world would have developed treatments for the symptoms of infectious diseases and remedies to eliminate microorganisms from their bodies. The codex of San Martin de la Cruz (1552) and that of Florentino de Sahagún (1530) constitute important sources of information. They contain clear descriptions of *A. lumbricoides* and *Toxocara canis* (Box 27.7) immediately following the conquest of Mexico; reference is also made to *E. vermicularis*. It has been possible to establish the use of plant infusions cooked by the Aztecs to produce, "medicine for killing worms" (Figure 27.4).

A recent report summarizes the place of current findings regarding ancient parasite therapy as part of an ongoing multidisciplinary approach [10].

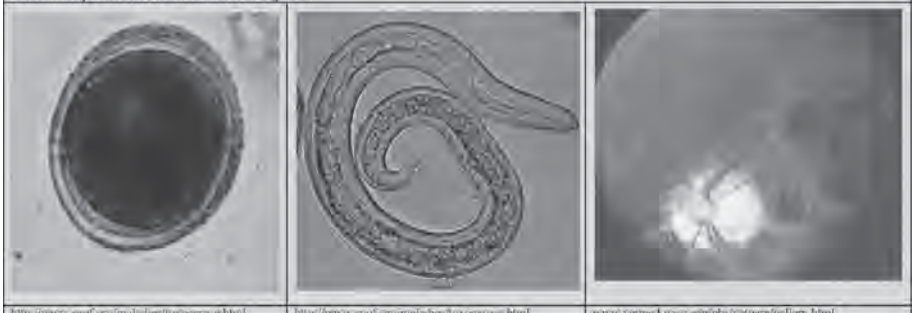
27.3 EPIDEMIOLOGY OF ANCIENT INFECTIOUS DISEASES

27.3.1 Smallpox

This disease has great historical importance, but its description by historians and chroniclers is not sufficiently specific for diagnostic purposes. Fornaciari and Marchetti [15] used

Box 27.7. TOXOCARIASIS

Our ancestors acquired parasites from animals, creating the first infectious diseases, these being the same as those affecting us today. The ability to infect humans was possible because these organisms were not completely specialized in their relationships relationships with their hosts. See color plates.



http://www.anof.org/maladies/toxocarose.html

Egg *Toxocara canis*
Infection in dogs has a worldwide distribution and infected adults excrete few eggs. Dogs are infected by ingesting the eggs from soil. The main source of infection is puppies, which excrete large numbers of eggs.

http://www.anof.org/maladies/toxocarose.html

Larve de *Toxocara canis*
In man, who is not the normal host, the eggs hatch in the stomach and second stage larvae penetrate the mucosa to enter the circulation reaching the intestinal viscera and liver and may pass into the general circulation through the lungs and end up in the brain, eye and other organs.

www.vetmed.wsu.edu/pfs/vetpath/gallery.html

Larva of *Toxocara* in the human eye
Human infection is acquired by children playing in contaminated soil on playgrounds. The penetrating larva has become encysted, leading to the formation of a large granuloma, seen here at the posterior pole of the fundus.

Fig. 27.4. Aztec herbalists' original sketches show plants used as antihelminthics which remain unidentified today [11,41].

immunohistological methods to demonstrate the presence of this virus in a medieval mummified skin lesion (Box 27.8).

27.3.2 Chagas Disease

Fornaciari et al. [14] used the same method as in smallpox (above) to demonstrate the presence of *T. cruzi* amastigotes in the wall of a dilated esophagus in an Andean mummy.

27.3.3 Malaria

This disease does not produce specific diagnostic changes grossly recognizable in mummified bodies nor in skeletons. Hence, immunogical tests to identify a histidine-rich protein secreted by *P. falciparum* (Box 27.9) have been employed. While an ELISA technique exists for use on blood, a modification of this method for field tests marked under the brand name "ParaSight-F" test has been used on human mummified tissue. Positive results have been reported on Egyptian mummies [32]. However, other authors have reviewed the evidence for that particular method and feel [42], ". . . immunological methods are not yet sufficiently reliable for routine use in this field."

27.3.4 Influenza Virus

In 1997, the United States Armed Forces Institute of Pathology in Washington, DC, recovered the 1918 "Spanish" influenza DNA from a 1918 autopsy lung sample that had been embedded in paraffin, and from lung tissue of an Alaskan 1918 influenza victim [48]. In 2004 other researchers identified the hemagglutinin gene whose protein product was responsible for the extraordinary virulence of that 1918 influenza virus [28].

Detailed information is given in Chapter 13.

27.3.5 Tuberculosis

Although the diagnosis of tuberculosis has been made on the basis of gross morphologic changes in ancient skeletons, such alterations can be simulated by other agents. Spigelman and Emma [46] described a molecular method to identify an aDNA sequence unique to the tuberculosis "complex," and Salo et al. [43] employed this technique to identify the presence of *M. tuberculosis* in the lungs of a 1000-year-old female Andean mummy, establishing the presence of this disease in South America 500 years prior to European contact. Additional information is given in Chapter 5.

27.3.6 Leprosy

The gross skeletal lesions of leprosy can be even more problematic diagnostically than those of tuberculosis. Hence, specific identification by molecular biology methodology [23, 38] in ancient bone has been particularly welcome. See Box 27.9.

27.3.7 Plague

Plague is a human infection by the bacterium *Yersinia pestis*. The reservoir lies in feral rodents that pass on the infection to domestic rodents. The vector is a flea (*Xenopsylla cheopis*) that parasitizes rats (*Rattus rattus* and *R. norvegicus*). The flea transmits the disease to humans when it disgorges the bacteria into the wound during a blood

Box 27.8. SMALLPOX (VARIOLA)

(also known by the Latin names *Variola* or *Variola vera*) is a highly contagious disease unique to humans. It is caused by two virus variants called Variola major and Variola minor. V. major is the more deadly form, with a typical mortality of 20–40 percent of those infected. The other type, V. minor, only kills 1% of its victims. Many survivors are left blind in one or both eyes from corneal ulcerations, and persistent skin scarring – pockmarks – is nearly universal. Smallpox was responsible for an estimated 300-500 million deaths in the 20th century. As recently as 1967, The World Health Organization (WHO) estimated that 15 million people contracted the disease and that two million died in that year.

In individuals without immunity, smallpox infection can be a devastating disease. Susceptible modern Europeans still suffer mortality rates of 25-50%. Post-Columbian contacts of Europeans with native North American groups may have caused 80 to 90 percent mortality. The exaggerated impact on such populations probably resulted from social factors (e.g., community collapse when the entire population was afflicted simultaneously, etc.). The American Civil War soldier in Fig. 3 and the adult female (Fig. 4) demonstrate an extreme degree of skin eruption. Photos from the National Museum of Health and Medicine, Washington, D.C., courtesy Paul Sledzik.

Box 27.9. MALARIA

The ancestors of present-day man (*Homo sapiens sapiens*) appeared in East Africa some 3.5 mya (*Australopithecus*) and then migrated to Europe, Asia, and later to the Americas. The first human migrations were accompanied by the three human plasmodia (*Plasmodium falciparum, P. vivax and P. malariae*) imported from Africa into the Mediterranean region, but it was the Neolithic revolution which created actual malarial foci.

Grupo de Parasitología INS – Bogotá.

***Plasmodium falciparum*:**
(Blood film, wet mount, x1000 under oil immersion). Photomicrograph of fine rings *Plasmodium falciparum* trophozoites. *P. falciparum* is the most common species in tropical and subtropical areas and gives rise to 'malignant tertian malaria', so –called because severe, often lethal complications can develop.

http://www.lshtm.ac.uk/prospectus/images/howto/Anoph_gambiae-ITDMRC.jpg

Anopheles gambiae
Malaria is transmitted by female *Anopheles* mosquitoes. Most species bite indoors at night but some are outdoor feeders.

Courtesy Prof CJ. Mannikelle. CIMPAT –Universidad de los Andes , Bogotá , Colombia

Gross section of brain in cerebral malaria
Cerebral malaria results when cerebral capillaries are blocked by erythrocytes containing developing falciparum schizonts.

meal. In the human the bacteria are drained by lymph channels into lymph nodes where they generate abscesses (the "bubonic" form), reach the blood ("septicemic" form), and extend to other viscera including the lung ("pneumonic" form).

Many ancient epidemics have been attributed to this bacillus, such as the "Justinian plague" (A.D. 542-3) in Constantinople and the "Plague of Athens" in 430 B.C. [6,47] that carried a high mortality, but these are not reported with sufficient detail to establish the etiology with certainty. The most major epidemic ("the Black Death") whose victims manifested buboes and other features of this illness began in Central Asia in A.D. 1346 and in the subsequent 4–5 years followed international trade pathways, sweeping through Africa, Middle East, and Europe. As many as half of the world's population (60 million people?) may have perished, with devastating social, economic, and demographic impact. The responsible bacillus was isolated in Hong Kong in 1894 [4].

No ancient human remains bearing the gross stigmata of plague have been reported. aDNA methods successfully identified the molecular targets for *Y. pestis* in three midfourteenth century adult and children's skeletons (teeth) from southern France [39]. Similar methods applied to teeth from two sixth century adults from northern Bavaria resulted in identical results [51]. Thomas et al. [50], however, made exhaustive efforts with multiple DNA targets and methods on skeletal remains from multiple European plague burial sites on 108 teeth from 61 individuals without identifying *Y. pestis* by these methods.

27.3.8 Treponematosis

The lack of satisfactory specificity of skeletal changes due to treponemal infections has hindered our understanding of this disease. The study reported by Kolman et al. [29] provided great promise for a solution to this problem. They extracted aDNA from a 200-year-old bone from Easter Island showing gross lesions expected in treponematosis. This aDNA exhibited a single-base polymorphism that distinguished *Treponema pallidum* from other treponemas. They also extracted from the same bone an antibody that had a strong affinity for the *T. pallidum* antigen. This work, however, has not yet been reproduced in independent laboratories.

27.4 CLUES REGARDING AMERICAN HUMANS

Some parasite organisms' evolution and dispersion were continuous processes which diverged when the continental masses separated from Pangaea, the super-continent, around 100 mya. At this time, ecological niches began to be found and well-defined host–parasite relationships created. Efficient dispersion methods were achieved and in some cases were included in the life cycles of various different hosts. This process took thousands of years to become established in the American continent. It may well have been at a point of equilibrium which became disrupted when the continent's first human settlers arrived and interacted with the environment.

Our ancestors acquired these parasites from animals, creating the first infectious diseases, these being the same as those affecting us today. The ability to infect humans was

possible because these organisms were not completely specialized in their relationships with their hosts. On the contrary, they have a certain degree of plasticity, because the parasites' morphological and physiological variety may allow them to have access to a wide range of hosts.

Some theories regarding the beginning of American cultures suggest that America was a continent that was free of parasites and that the infectious agents must have reached the New World with the European colonizers during the time of the conquest. However, Reinhard [40] showed that various autochthonous parasites from America greeted the first men to reach the New World by giving them debilitating and mortal diseases.

Examining diverse human remains (Alaskan seal hunters, prehistoric cave painters in North America, and Inca farmers in Peru) has led to finding a group of parasites belonging to America. Some of these parasites were able to resist the cold confronting the first human immigrants who came from the Old World across the Bering Straights during the Pleistocene Age, a long time before the arrival of Europeans in America.

27.4.1 The First Inhabitants

Parasite organisms' establishment, dispersion, and evolution on the American continent were part of a continuous evolutionary process. The same happened to all animal species sharing habitats on the separated continents or which became endemic species occupying specific niches and keeping fairly well-defined host–parasite cycles. It is also important to bear in mind that many parasite entities are transmitted by insect vectors when these are feeding on the blood of wild animals and thus making them reservoirs. These transmission cycles took thousands of years to become established and, in most cases, a host–parasite equilibrium became well established which only became altered when man started to intervene in the environment as he began to build dwellings, hunting, and acquiring energy-providing resources for his subsistence.

The origin and dispersion of humans in the New World coincided with the last part of the Pleistocene glaciations and such distribution was influenced by these glaciations in very different ways. The process periodically became accelerated when the Bering Straits narrowed sufficiently to allow humans to cross towards the American continent.

Archaeological excavations have been made from the Aleutian Islands, the western coasts of the United States, and Canada down toward the coasts of Mexico and Central and South America. The closest evidence regarding the presence of human activities provides data from around 15,000 years ago, probably 10,000–12,000 years ago when human settlements' activities began in South America. Significantly older dates from a few sites remain controversial.

Various species of parasites had enough time to establish themselves and infect the first humans who trod the New Continent. In turn, other species were passively transported by these new visitors before beginning to find new shelter and refuge in other animal species.

Closer contact was established between man and animals when many of them became domesticated in the vicinity of human dwellings and their peridomiciliary areas where sowing and general agricultural activities were carried out. The continents became reconnected when transoceanic voyages became frequent around the sixteenth century, resulting in rapid global colonization. This was enhanced by human diversity, and when their domestic animals and plants were

Box 27.9. LEPROSY

sometimes known as **Hansen's disease**, is an infectious disease caused by *Mycobacterium leprae*, an aerobic, acid fast, rod-shaped mycobacterium. The modern term for the disease is named after the discoverer of the bacterium, Gerhard Armauer Hansen. Sufferers of Hansen's disease have historically been known as **lepers**, however this term is falling into disuse as a result of the diminishing number of leprosy patients and the pejorative connotations of the term. The terms "leprosy" and "lepers" can also lead to public misunderstanding because the Bible uses these terms in reference to a wide range of incurable skin conditions other than Hansen's disease.

The photo is characteristic of facial features seen in lepromatous leprosy. The swollen areas are delimited by the sunken lines generated by underlying soft tissue necrosis. The ulcerative lesions of the nasal and oral mucosa frequently extend into the underlying bone. In paleopathological specimens erosion of the anterior nasal spine is common noted. Particularly prominent is anterior maxillary destruction with loss of the anterior teeth, a combination of findings termed the "rhinomaxillary syndrome" (earlier called "facies leprosa"). [4] Photo from G.A. Hansen (1895), [23]

added to local wide plants and feral animals. Similar introduction and extinction processes continue today in spite of modern public health control measures at frontiers.

27.4.2 The First Parasites

The main current evolutionary theories propose that life on earth began with parasitism, initially of some molecules parasitizing other molecules and thus opening the way for the formation of the first cells. This was followed by cells parasitizing other cells, leading to the diversification of molecular and cellular forms. These hypotheses base their suppositions on molecular evidence found in different levels of life and cellular organization. For example, short mobile DNA sequences have been observed (transposons) which are inserted into cell DNA by enzyme action, thereafter being considered DNA parasites. Molecules like transposons have allowed researchers to establish a strong relationship between parasitism and the evolution of life on earth. A good example of how life has evolved – thanks to parasitic relationships – lies in the appearance of chloroplasts and mitochondria. These structures (originally being small parasite cells from larger cells) supposedly became eukaryote cell organelles. This type of finding has led to the concept of parasitism being reevaluated; a parasite is currently defined as being any life form or organic compound capable of multiplying itself which finds its home (ecological niche) in another form of life. This mechanism has been so important in the history of life that it is estimated that there are ten times more parasite species in nature than those that are extra cellular [1].

27.4.3 The First Infectious Diseases

The origin, dispersion routes, and impact of microbes in the pre-Columbian New World have been described including *Treponema* and the measles virus, as well as diseases caused by parasite protozoa such as *Plasmodium* and *Trypanosoma*. The paleoepidemiology of diseases caused by helminthes has also received special mention because of their wide distribution. Information regarding the fauna of New World helminthes has been principally obtained from paleofaeces and mummified bodies. It is quite probable that some helminth shared by humans, domestic, and wild animals had a circum-Arctic distribution or were common to the two continents.

Trichinosis and toxocariasis are clear examples. However, only *E. vermicularis* and *T. trichura* have been well documented amongst the strictly human parasites, as well as *Ancylostoma duodenale*. The latter could have been transferred from Asia to America, even though there seems to be no evidence to confirm this. It is thus most important to make use of very sensitive and specific techniques to resolve these questions.

Our ancestors' feeding habits caused them serious health problems. The habit of eating meat and the lack of combustibles for cooking it in the Arctic allowed the first Americans to become infected with *Trichinella* genus tapeworms which, in great numbers, can cause agonizing muscular pains as well as inflammation around the eyes, hemorrhages under the nails, and even death. The first nematode infections produced by *Trichinella nativa* species occurred by consuming uncooked polar bear meat by the first Americans; this species' cysts infect the polar bear.

The way in which they became infected was also due to their alimentary habits. The bear eats the eggs of birds which, in turn, have eaten rodents infected with the parasite. Due to their rapid metabolism on defecating, the birds expel pieces of undigested rodent meat containing parasite's cysts, frequently on top of their eggs. The small tapeworms from the *Trichinella* genus invaded the muscles and even the hearts of early Americans. Tapeworms belonging to Acanthocephala perforated the intestinal walls of the ancient inhabitants of the great basin of present day United States.

Protozoa belonging to the *Leishmania* genus ulcerated throats and mouths in the Andean region inhabitants. Other protozoa belonging to the *Trypanosoma* genus caused cardiac damage and provoked deformations in their victims' digestive tracts and sudden deaths due to cardiac failure.

27.5 NEW PERSPECTIVES

The search for answers concerning the presence of certain parasites in the New World has led to new approaches being formed about the peopling of America. This is the case of *Uncinaria*, a nematode genus causing a type of anemia, affecting humans in America before any contact was established with the conquistadores. Even though nematodes from the *Enterobius* genus do not cause mortal infections, they do produce severe discomfort for their hosts (even today), having accompanied man since his origins in Africa. These parasites have been found in remains being at least 10,000 years old.

The presence of these parasites in pre-Hispanic samples has generated great controversy because, as they are tropical parasites (endemic for equatorial regions), they certainly could not have made the crossing of the Arctic due to the low temperatures. In an attempt to explain these observations it has been proposed that the presence of these parasites is evidence of transpacific contact between American and Asian cultures.

Another possible explanation recently advanced for these parasites' presence in the New World has emerged from a biological phenomenon known as hypobiosis, consisting of organisms passing long periods of time in a latent state during which they can slow their metabolism until conditions become optimum again. This phenomenon has been observed in *Uncinaria* and very possibly could have allowed these organisms to survive trans-Bering migration [36].

Various parasites from animals which were present in America before the arrival of man during the Pleistocene age adapted to the presence of extremely curious, meddling new primates, leading to establishment of parasitic relationships with them.

This is the case of some Platyhelminthes, which can measure up to 5 m in length, lodging in their hosts' intestine. They belong to the *Diphyllobothrium* genus, existing in America long before the arrival of man and found as parasites of ani-

mals, including several fish and the *Otaria byroni* (sea-lion) as final host. Because of uncooked fish consumption by recently arrived humans they became alternative final hosts, suffering sometimes severe and debilitating disease, including pernicious anemia (a type of anemia related to vitamin B12 deficiency). This parasite–animal–man relationship has led to new ideas being advanced to explain the evolution of parasites and their broad capability to adapt to new hosts.

ACKNOWLEDGMENTS

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ABBREVIATIONS

aDNA: Ancient deoxyribonucleic acid
DNA: Deoxyribonucleic acid
ELISA: Enzyme-linked immunosorbent assay
PCR: Polymerase chain reaction
IFAT: Immunofluorescence antibody detection test
rRNA: Ribosomal ribonucleic acid
rDNA: Ribosomal deoxyribonucleic acid
RIA: Radio-immuno assay
mya: Million years ago

GLOSSARY

Anthracosis: Usually asymptomatic form of pneumoconiosis caused by deposition of coal dust in the lungs.

Archaeology: (from the Greek words αρχαίος = ancient and λόγος = word/speech/discourse) It is the study of human cultures through the recovery, documentation, and analysis of material remains and environmental data, including architecture, artefacts, biofacts, human remains, and landscapes.

Canopic: Among the ancient Egyptians, canopic jars were covered funerary vases, normally composed of clay, intended to keep the viscera of mummified corpses. All the viscera were not kept in a single canopic jar, but rather each organ in its own.

Cesspit: It is a pit, or covered cistern, which can be used for refuse or sewage.

Coprolites: Fossilized excrement of animals. Under the right conditions animal feces have been fossilized (preserved) are called coprolites.

Dispersion: Biological dispersal, refers to those processes by which a species maintains or expands the distribution of a population. Dispersal implies movement – movement away from an existing population (population expansion) or away from the parent organisms (population maintenance).

DNA probe: A hybridization probe is a short piece of DNA (on the order of 100–500 bases) that is denatured (by heating) into single strands and then radioactively labeled, usually with phosphorus (32P or 33P). This creates a short piece of radioactively labeled DNA with known sequence that will hybridize with any complementary nucleic acid strands.

Epidemiology: It is the study of the distribution and determinants of disease in human populations, and the application of this study to control of health problems.

Epizootiology: A science that deals with the character, ecology, and causes of outbreaks of animal diseases.

Ethnography: (from the Greek ethnos = nation and graphein = writing) Refers to the qualitative description of human social phenomena, based on fieldwork. Ethnography is a holistic research method founded in the idea that a system's properties cannot necessarily be accurately understood independently of each other.

Genome: It is the whole hereditary information of an organism that is encoded in the DNA (or, for some viruses, RNA). This includes both the genes and the noncoding sequences.

Geology: (from Greek γη- (ge-, "the earth") and λογος (logos, "word", "reason")) It is the science and study of the Earth, its composition, structure, physical properties, history, and the processes that shape it. It is one of the Earth sciences.

Glaciations: A glaciation (a created composite term meaning Glacial Period, referring to the Period or Era of, as well as the process of high glacial activity), often called an ice age, is a geological phenomenon in which massive ice sheets form in the Arctic and Antarctic and advance toward the equator.

Host: In parasitology, an intermediate host is an organism that is infected with a parasite that will not reproduce sexually within it, while a definitive host is one in which the parasite reproduces.

Kinetoplast: It is a specialized region within the mitochondrion containing the large amount of mitochondrial DNA characteristic of trypanosomes.

Mortality: Mortality rate (the word mortality comes from mortal, which originates from Latin mors, death) is the annual number of deaths (from a disease or at general) per 1000 people. It is distinct from morbidity rate, which refers to the number of people who have a disease compared to the total number of people in a population.

Neolithic: (Greek neos = new, lithos = stone, or "New Stone Age")A period in the development of human technology that is traditionally the last part of the Stone Age. The term is more commonly used in the Old World and its application to cultures in the Americas and Oceania is problematic.

Palynology: It is the science that studies contemporary and fossil palynomorphs, including pollen, spores, dinoflagellate

cysts, acritarchs, chitinozoans, and scolecodonts, together with particulate organic matter (POM) and kerogen found in sedimentary rocks and sediments.

Phylogeny (or phylogenesis): It is the origin and evolution of a set of organisms, usually of a species. A major task of systematics is to determine the ancestral relationships among known species (both living and extinct), and the most commonly used methods to infer phylogenies include cladistics, phenetics, maximum likelihood, and Bayesian.

Pleistocene: The Pleistocene Epoch is part of the geologic timescale, usually dated as 1.8–1.6 million to 10,000 years before present, with the end date expressed in radiocarbon years. It covers most of the latest period of repeated glaciation, up to and including the Younger Dryas cold. The end of the Younger Dryas has been dated to about 9600 B.C. (11550 calendar years B.P.).

Paleontology: It is the study of the developing history of life on earth, of ancient plants and animals based on the fossil record, evidence of their existence preserved in rocks.

Parasite: A parasite is an organism that lives in or on the living tissue of a host organism at the expense of that host. The biological interaction between the host and the parasite is called parasitism. Parasitism is a type of symbiosis, by one definition, although another definition of symbiosis excludes parasitism, as it requires that the host benefit from the interaction as well as the parasite.

Prevalence: The prevalence of a disease in a statistical population is defined as the ratio of the number of cases of a disease present in a statistical population at a specified time and the number of individuals in the population at that specified time.

Reservoirs: Refers to the carrier of a virus or parasite for which they are not pathogenic.

Scatology: In medicine, biology, and paleontology, is the study of feces. Scatological studies allow one to determine a wide range of biological information about a creature, including its diet (and thus where it has been), healthiness, and diseases such as tapeworms.

Tandem: This is a term from genetics, which describes a pattern that helps determine an individual's inherited traits. Tandem repeats and variable number tandem repeats in DNA are two or more nucleotides in a pattern which are directly adjacent to each other.

Transcription: It is the process through which a DNA sequence is enzymatically copied by an RNA polymerase to produce a complementary RNA.

Transposons: Sequences of DNA that can move around to different positions within the genome of a single cell, a process called transposition. In the process, they can cause mutations and change the amount of DNA in the genome. Transposons are also called "jumping genes" or "mobile genetic elements."

Vector: An organism that does not cause disease itself but which spreads infection by conveying pathogens from one host to another. Species of mosquito, for example, serve as vectors for the disease-causing West Nile Virus; which the insects may ingest by feeding from an infected bird and regurgitate into a human, infecting him or her. This sense of "biological vector" is the primary one in epidemiology and in common speech.

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CHAPTER 28

Insights Into Structure and Evolution of Bacterial Species That Are Revealed by Molecular Methods

P. Roumagnac,[1] L. Gagnevin,[2] O. Pruvost,[2] and M. Achtman[1]

[1]*Department of Molecular Biology, Max-Planck-Institut für Infektionsbiologie, Schumannstrasse 21/22, 10117 Berlin, Germany*

[2]*CIRAD, UMR Peuplements Végétaux et Bioagresseurs en Milieu Tropical CIRAD-Université de la Réunion, Pôle de Protection des Plantes, 7, chemin de l'Irat, 97410 Saint Pierre, Réunion Island, France*

28.1 INTRODUCTION

In recent decades, microbiologists have been confronted with a variety of unanticipated problems: worldwide emergence of drug resistance, difficulties in predicting the success of novel vaccination strategies, global changes in pathogen distribution, ongoing plant diseases, and the threat of bioterrorism. Solving these problems will require molecular data that have been acquired within a clear population genetic and evolutionary framework. In this chapter, we review the types of data that have been used to discriminate between strains within bacterial species and population genetic structures that can account for these differences between strains.

Diverse molecular methods have been used by microbiologists for the characterization and classification of microbial isolates (Fig. 28.1). We first review this historical development over the last five decades in order to place the current genome-typing era in a proper context. Experiments on molecular evolution became possible after 1952 when the sequences of insulin [201] and other proteins yielded the first insights regarding how species differ at the molecular level.

Two crucial models of molecular evolution were developed during this period: The molecular clock hypothesis asserts that amino acid differences between taxa accumulate at a constant rate per year [256]. The neutral mutation hypothesis [120,122] argues that most mutations are not under selection pressure and accumulate with time due to random drift [121]. These concepts and the controversies they stimulated led to multiple analyses of intra- or interspecific polymorphism, as well as initial attempts to derive phylogenetic trees based on molecular data (see Chapter 17, which presents a superb review of phylogenetic concepts and methods).

Protein sequencing is too labor-intensive to provide a methodology for screening phenotypic diversity among multiple individuals. However, protein sequence diversity is accompanied by changes in serological properties and electrophoretic charge that are suitable for large-scale screening. Large datasets of eukaryotic protein diversity were accumulated in the 1970s by such methods, which in turn stimulated the development of novel algorithms for quantitative analysis that were suitable for computer-based analyses, including genetic distance measures [36,167] and clustering algorithms [214].

These new evolutionary and population genetic approaches were largely restricted to eukaryotic organisms until the end of the 1970s. Although serotyping and biochemical typing were used routinely for the definition of bacterial species, population genetic theories to account for this diversity were lacking until Selander and his colleagues applied multilocus enzyme electrophoresis (MLEE) to *Escherichia coli* and showed that particular combinations of alleles (electrophoretic types, ETs) were significantly more common than would be expected for a panmictic organism [208]. These ETs were considered to represent bacterial clones, a concept that was developed to account for the

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

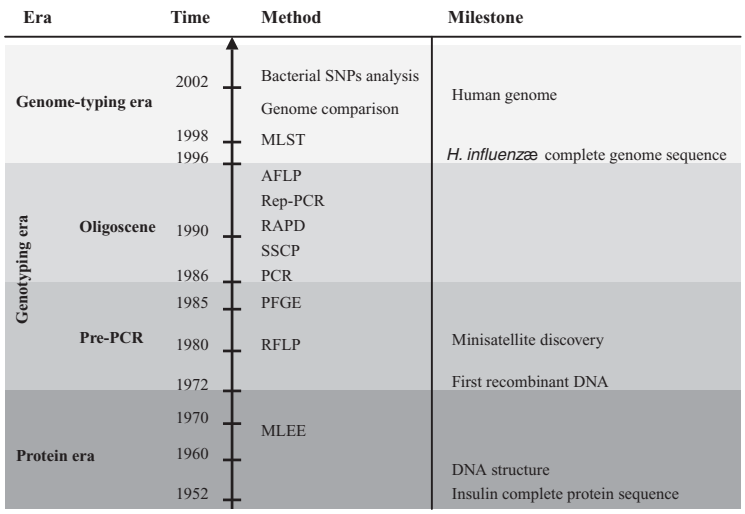

Fig. 28.1. Historical development of molecular methods over the last five decades.

global spread of FIRN *Salmonella typhimurium* [176], a particular association of particular rare serotypes of *E. coli* with gastroenteritis [180] and the subdivision of most *E. coli* K1 isolates into a limited set of clones with uniform outer membrane protein patterns and biotypes [4]. Subsequent analyses by Selander and his colleagues with a large variety of bacterial species revealed that many isolates belonged to a limited number of ETs or clusters of related ETs, called clonal complexes [206,207].

DNA sequencing was still too difficult at the time to compete with protein-based techniques such as MLEE. However, the invention of the polymerase chain reaction (PCR) [163] has revolutionized the analysis of genetic diversity because it allows segments of DNA to be amplified and sequenced rapidly from minuscule amounts of cells or chromosomal DNA. Extensive access to DNA sequence data has completely changed microbial population genetics, both qualitatively and quantitatively, and has provided a solid basis for understanding long-term evolutionary processes. One consequence is that the clonal concept for bacterial population structure became questionable after seminal publications in 1993 provided evidence for frequent recombination within bacterial populations [151,171]. Numerous examples of recombination have now been described, even among bacteria that were thought to be highly clonal, such as *Salmonella enterica* [32]. A second consequence, particularly once complete genome sequences became available, is that bacterial genomes are now thought to be much more plastic, in part due to horizontal gene transfer between unrelated species [149,173].

The distinction between clonality and recombination is crucial to our thinking about bacterial population structure. But this distinction is not adequate to capture all the nuances in population structures that would be expected to differentiate bacterial pathogens due to different modes of transmission and evolutionary history. Furthermore, within single bacterial species, the population structures need not be the same for all organisms, for example, *Neisseria meningitidis* where frequent recombination [68,105] does not preclude the existence of clonal groupings such as serogroup A [18]. Even in the presence of extensive recombination, geographical barriers and historical isolation can result in well-defined groupings, such as in *Helicobacter pylori* [3,63]. Thus, one appropriate goal for molecular epidemiology might be to define stable groups of organisms with characteristic epidemiological patterns even within species where other groupings are only ephemeral [52,104,230]. Such durable classification schemes should probably be based on top-down methodologies that can differentiate all isolates within a bacterial species. For short-term epidemiology, it may not be possible to achieve sufficient resolution with classification schemas and it may be more appropriate to use bottom-up typing schemes even if they have limits for global classification (Fig. 28.2).

In the first part of this chapter, we review currently available methods for indexing bacterial sequence variation in chronological order. In the second part, we focus on scientific questions and how they have been addressed by various methods.

28.2 METHODS THAT INDEX DNA POLYMORPHISM

DNA sequence variation can be assessed by a variety of methods, which we categorize here as direct sequencing and "fingerprint"-based methods because these tend to be evaluated differently. Extensive use of fingerprinting flourished in the 1990s, primarily as a tool for typing isolates that were collected over short time periods from local outbreaks (local epidemiology). DNA fingerprinting may remain the method of choice for forensic determination of the relationships of isolates within single source outbreaks. However, as sequencing

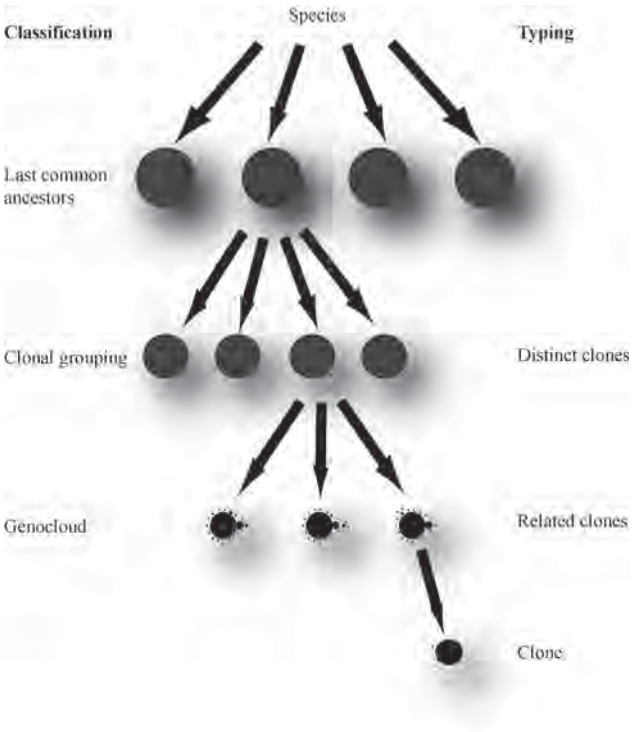

Fig. 28.2. Classification versus typing. Classification (left) is a top-down strategy, which subdivides species into clonal groupings, each potentially representing the descendants of last common ancestors. Clonal groupings can sometimes consist of multiple genoclouds, each of which contains a frequent genotype (larger circle) plus rare variants (dots) [255]. Different genoclouds from one clonal grouping may exist in different countries or at different time points. Typing (right) is a bottom-up strategy, which can assign very closely related variants to so-called "clones" and can potentially recognize that genoclouds within a clonal grouping are related. Typing can only rarely elucidate the phylogenetic relationships between different clonal groupings (from Achtman [2]). Permission has been obtained.

technology has improved and become both cheaper and more readily accessible, more and more results are now being obtained by direct sequencing. Sequences are eminently suitable for phylogenetic comparisons and the results are readily comparable between different laboratories. However, it is currently unclear whether sequencing can replace fingerprinting for a general use.

Molecular markers may be divided in two main groups: neutral markers and contingency (or "outlier") markers. Sequence diversity between neutral markers reflects genetic drift and can differ between populations that are ecologically or geographically separated. Although neutral markers should be relatively independent of selective pressures, they can be influenced by selection on genetically linked loci, and unusual phylogenetic patterns have been attributed to hitchhiking with neighboring genes that are under diversifying selection [59]. However, in the absence of selection, neutral markers should simply reflect the demographic and evolutionary history of bacterial populations. Contingency markers react to changes in the environment and allow bacterial adaptation to unusual conditions. They may be recognized as "outliers" with patterns of variation that are divergent from the rest of the genome [143] and include loci associated with pathogenicity, fitness, environmental adaptation, and speciation. Loci with high mutation rates, such as some minisatellites, are probably also contingency markers.

28.2.1 DNA Fingerprinting

DNA fingerprinting is based on scoring the presence or absence of multiple DNA fragments of different sizes that reflect sequence polymorphisms within bacterial genomes. In some cases, the fragments are generated by restriction endonucleases whereas in others they are generated by specialized PCR amplification designed to result in a limited number of fragments. Limited numbers of fragments can be detected by direct measurements while specific fragments are usually detected by hybridization of genomic DNA with specific probes. A plethora of techniques has been described that implement variations on this general strategy. In order to maintain a phylogenetic perspective, these will be summarized in terms of whether they concentrate on neutral or contingency markers. Microarray techniques use different technological approaches and are treated subsequently.

28.2.1.1 Methods based on neutral markers Some fingerprinting methods assay variation in restriction endonuclease sites, including restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP). These will be described as one group before describing methods that assay variation in repetitive sequences, such as randomly amplified polymorphic DNA (RAPD); also called arbitrarily-primed PCR (AP-PCR), repetitive element-based PCR (rep-PCR), insertion sequence RFLP (IS-RFLP); detection of IS elements by PCR (IS-PCR), or insertion sequence-ligation mediated-PCR (IS-LM-PCR). Some of these methods can produce hundreds of polymorphic markers, which are randomly distributed around the genome and might be expected to correspond to neutral markers. However, 15/306 AFLP loci that were genotyped in intertidal snails were inferred to be under selection [247], possibly due to genetic linkage to other loci that are under selection.

RFLP [29]: DNA is first digested to completion with a restriction enzyme, yielding fragments that usually range from hundreds of base pairs up to 20 kb (Fig. 28.3).

The DNA fragments are size-fractionated by agarose gel electrophoresis. Due to the large numbers of fragments, specific fragments are normally detected by hybridization. According to the Southern blotting procedure [216], DNA fragments are denatured within the gel by alkali and transferred to a nitrocellulose or nylon membrane. The membrane is then hybridized with a specific nucleic acid probe that can be readily detected because it is radioactive or fluorescent. Mutations that alter former restriction sites result in the fusion of two fragments into one longer fragment. Conversely, mutations that generate new restriction sites convert one

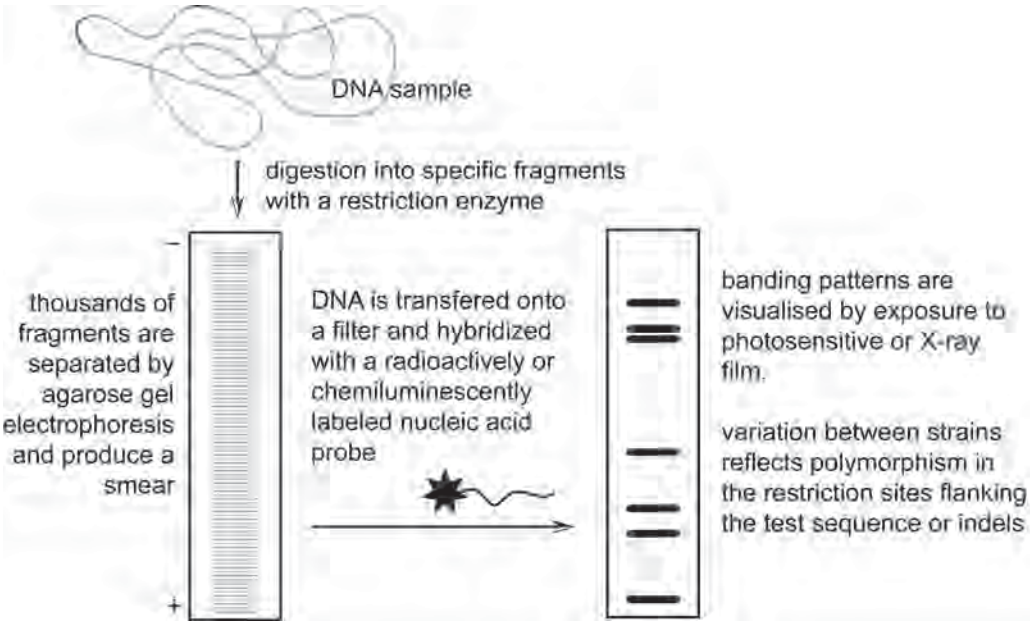

Fig. 28.3. Southern blotting to detect RFLP.

fragment into two shorter fragments. RFLP fragment patterns may also change due to indels (insertions or deletions), in which case the number of fragments remains the same but the sizes of individual fragments are altered. Visualization of hybridizing bands is performed with X-ray film for a radioactive-labeled probe or by image analysis for light emitting probes.

RFLP with probes containing ribosomal RNA sequences, so-called ribotyping, has been widely used over the last decades. Ribotyping is highly reproducible [219] and can be a very sensitive method for detecting changes within species where genomic rearrangements involve rRNA operons, such as *Salmonella* Typhi [11,56,133,139,168]. Ribotyping has also provided reproducible typing of *Yersinia pestis* (83), *Vibrio cholerae* [113,128], *N. meningitides* [237], *Shigella* [195], *H. pylori* [225], *Bacillus anthracis*, and *Bacillus cereus* [209] or nosocomial pathogens [23].

PFGE [205]: PFGE allows the electrophoretic separation of large fragments up to several Mb in size [44,90] due to pulsed, alternating, and orthogonal electrical fields [5]. When combined with digestion with low-frequency cleaving enzymes in agarose, PFGE allows the analysis and mapping of complete bacterial genomes [74,103,252], the analysis of genomic rearrangements and variation in large DNA islands [74,196] and has facilitated evolutionary and population studies in a number of species [43,71].

As both a preparative and an analytical tool, PFGE has played a fundamental role for the study of many pathogens. For instance, within *N. meningitidis*, this method has been used to generate chromosomal maps [49], allowed the cloning of large fragments [123], and provided a powerful tool for monitoring the global spread of epidemic clones [34,76]. PFGE has been designated the "gold standard" for bacteriological typing [177,226,232]. It is used at the national level in the United States by the Center for Disease Control and Prevention (CDC) for identifying bacteria isolated from humans and contaminated food products using standardized conditions and electronic databases [55,154].

AFLP [241]: AFLP is a relatively cheap, easy, fast, and reproducible method that can generate hundreds of informative markers [162], from any bacterial genome.

Approximately 50 ng of DNA is digested with two distinct restriction endonucleases. The digested genomic DNA is then ligated to double-stranded oligonucleotide adapters that are specific for each of the two restriction products and which prevent regeneration of the original restriction site after ligation (Fig. 28.4). Oligonucletoide primers that are specific to the adapter sequence plus the restriction site plus one to four subsequent arbitrary nucleotides are then used to PCR amplify the ligated DNA. The number of resulting PCR products depends on the number and sequence of the arbitrary nucleotides. The PCR products are then usually size-fractionated by acrylamide gel electrophoresis or by capillary electrophoresis and are visualized using fluorescence, radioactivity, or silver nitrate staining. AFLP yields predictable results. For instance, all 48 bands that were predicted from the complete genome sequence of *E. coli* K-12

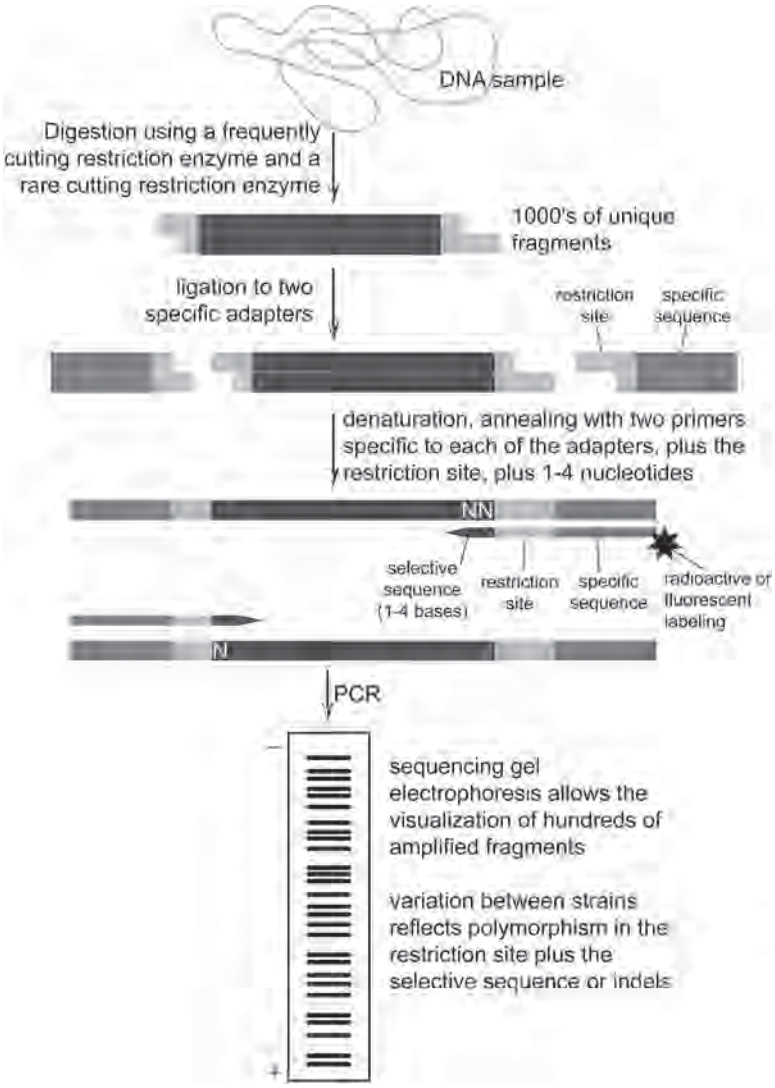

Fig. 28.4. The principles of AFLP.

were detected by AFLP and differed in size from the predictions by only 1 ± 2 bp [13].

AFLP has become an increasingly popular technique for genotyping bacterial species [202]. AFLP distinguished between isolates from *Campylobacter coli* and *Campylobacter jejuni* [46] and yielded a comparable phylogenetic tree to MLEE for the *E. coli Eco*R collection [13]. AFLP yields similar groupings to other molecular methods for a variety of bacterial species and is more discriminatory [14,53,102,106, 107,118,165,191,204].

However, in most analyses, the molecular basis for band differences remains unknown, resulting in data that can only be used to generate distance matrices rather than allelic data that can be used to trace evolutionary changes [162]. On occasion, it is also difficult to distinguish between comigrating fragments of similar molecular weights [160]. AFLP depends to a considerable extent on the most variable sequences within the genome, which may correspond to contingency markers whose patterns of diversity might overwhelm phylogenetic signals from neutral markers [2]. Possibly as a combination of these problems, AFLP seems to be most suitable for the rapid identification of major groupings within medium-sized sets of related isolates coupled with the recognition of particular probes that are characteristic of each of those groupings [15,98,233].

rep-PCR: Several interspersed repetitive sequence elements have been identified in prokaryotes, including repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), *Xanthomonas* repetitive intergenic consensus (XRIC), and BOX elements [81,250]. Although repetitive extragenic palindromic sequence probably plays a role in gene regulation [144], the functions of these particular

sequences remain unclear. Rep-PCR consists of PCR using primers for one of these repetitive elements and amplifies the intervening DNA between adjacent pairs of elements that are sufficiently close to be successfully amplified. The result is typically dozens of products of various molecular sizes that are size fractionated. Rep-PCR has been extensively used in identification and assessment of genetic diversity of pathogens [85,141,157]. Reproducibility within an individual laboratory can be high [112], but portability between laboratories remains problematical [51].

RAPD [248]: RAPD is based on low-stringency PCR amplification with single 10–20-mer oligonucleotide primers of arbitrary sequence.

The amplification products, corresponding to pairs of sites separated by less than a few kilo base pairs that happen to hybridize with the arbitrary primer used, are size fractionated (Fig. 28.5). RAPD yields PCR products of varying intensities that reflect variable sequence homology between the chromosomal sites and the primers. Therefore, even within a single laboratory, reproducible results can only be obtained by highly standardized protocols, including the use of the same thermocycler, and portability between laboratories is poor

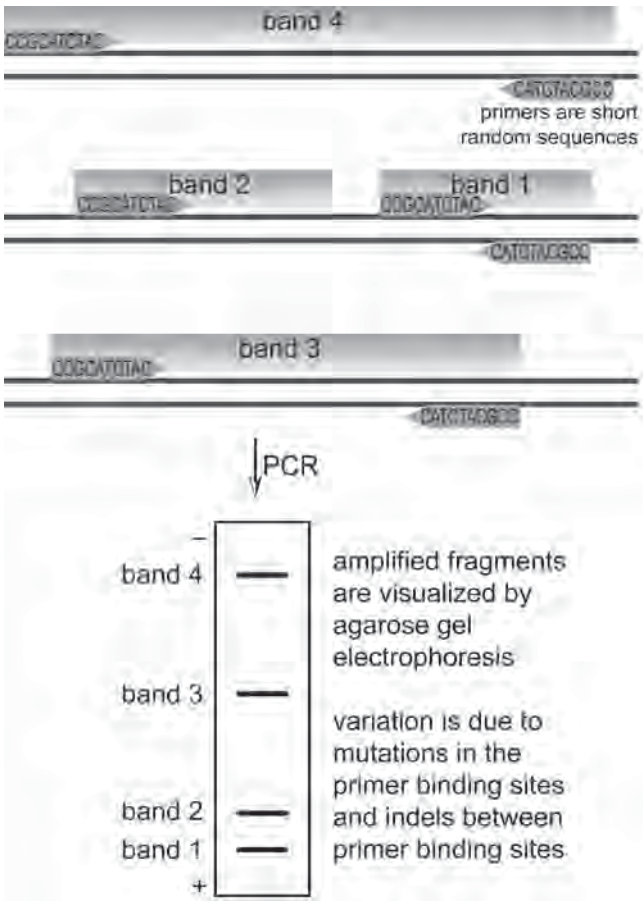

Fig. 28.5. The principles of RAPD using a hypothetical primer 5'-CCGCATCTAC-3'.

[185,203]. RAPD is an inexpensive, efficient, and sensitive method for recognizing genetic differences between closely related bacteria which was widely applied in the 1990s for diverse pathogenic organisms [7,9,136,238]. Even if RAPD has been largely been supplanted by other genotyping approaches, it is still widely used from a diagnostic perspective in the method variant called sequence-characterized amplified region (SCAR) [22,114,227,229].

IS-RFLP: Insertion sequences (ISs) are transposable DNA sequences that are usually less than 2.5 kb in length. Most ISs exhibit short terminal inverted-repeat sequences of between 10 and 40 bp and encode only functions involved in transposition [145]. More than 600 ISs have been identified from 171 bacterial and archaeal species [146], some of which fall into widespread families. Transposition of ISs can result in multiple, nearly identical copies within a genome which can then lead to plasmid [246] and chromosomal rearrangements [88]. These properties make RFLP analysis using particular ISs as a probe a valuable tool for investigating genetic diversity and molecular epidemiology.

IS-RFLP with the probe IS*6110* became the "gold standard" for large-scale molecular epidemiology of *Mycobacterium tuberculosis* [111]. However, it appears that IS*6110*-RFLP has many disadvantages. RFLP is time-consuming and requires live cultures and high-quality DNA. Some strains have few copies of IS*6110* or lack it [235]. Integration hotspots exist within the *M. tuberculosis* genome at which IS*6110* has inserted multiple times in independent genetic events (homoplasy), resulting in pseudo-clustering in unselected community-based studies [77,235]. Finally, because it is based on gel electrophoresis, matches between current fingerprints and historical fingerprints in databases require confirmation and extensive comparisons between multiple laboratories are difficult to perform.

Alternative IS typing methods based on PCR (IS-PCR, IS-LM-PCR) have been developed in the last decade [73,181,197]. IS-PCR is rapid, requires small amounts of DNA, and can be used with nonviable isolates [109,140,200].

28.2.1.2 Methods based on contingency markers

Bacterial genomes possess "contingency loci" that are extensively prone to gain and loss of function through slipped-strand mispairing and gene conversion [161]. Contingency loci allow pathogens to explore precipitous and unpredictable aspects of the host environment, while minimizing deleterious effects on fitness. The analysis of genetic variation in contingency loci of eukaryotes (microsatellites) has revolutionized their evolutionary and conservation biology, but these data also present problems that need to be considered [93]. Heterogeneity can be exceedingly high, even within populations, and homoplasies may be frequent. As a result, genetic distances between populations can quickly become very large, especially when populations undergo severe bottlenecks, resulting in statistically significant differences with little biological meaning [93].

Variable number of tandem repeats (VNTR) and MultiLocus VNTR analysis (MLVA): VNTRs, also called minisatellites,

are tandem repeats of 10 to >100 bp sequences [25]. Variation in the number of tandem repeats due to slipped-strand mispairing, polymerase inefficiency, and lack of DNA mismatch repair is a major cause of changes in contingency loci in both eukaryotes and prokaryotes [231]. During DNA replication, slippage due to mispairing between neighboring repeats can result in deletion or duplication of repeat segments [40]. Indels in VNTR loci occur with rates of 10^{-4} to 10^{-5} per nucleotide per generation in *B. anthracis* and even higher rates in other bacteria [116], whereas the spontaneous mutation for non-repetitive DNA is approximately 10^{-10} in *B. anthracis* and *E. coli* [54,240]. VNTR size variation can be detected by hybridization with probes specific for the repeat or flanking DNA. VNTR size variations can also be detected by PCR amplification of the entire locus with flanking primers (Fig. 28.6).

MLVA consists of determining the VNTR pattern at multiple loci [115]: Although population genetic analyses of micro- and minisatellites have been common for eukaryotes since the 1990s, MLVA was first used with bacteria in recent years. Mini- and microsatellites have now been identified in several bacterial species [31,60,84,131,132,152,192,222,228] and used for genotyping. VNTRs that have been used extensively in *M. tuberculosis* include the so-called direct repeat (DR, the basis of spoligotyping) and the poly(GC) rich sequences (PGRS) [110,211,243]. The DR region contains a variable

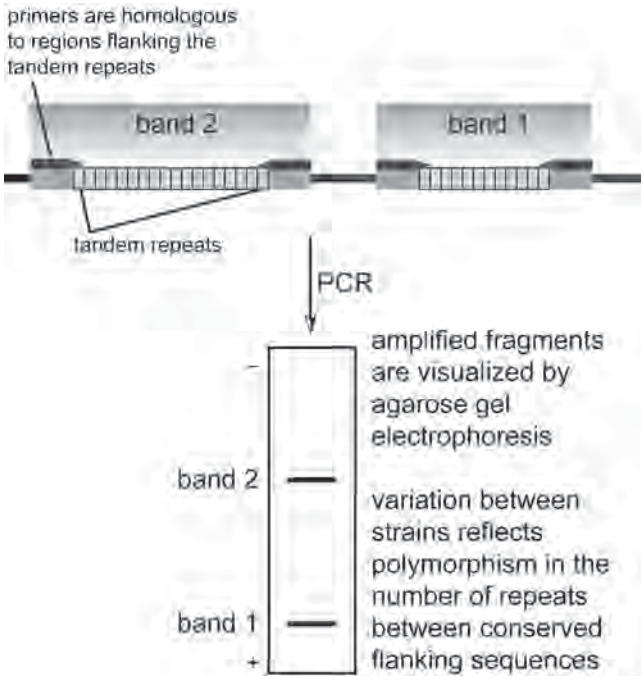

Fig. 28.6. The principles of PCR amplification of VNTR with flanking primers. VNTR fragment variation is detected by amplifying the segments containing different numbers of repeats by using primers flanking the segments for PCR. The polymorphic bands can be interpreted as the variation in the numbers of the tandem repeats.

number of short direct repeats interspersed with non-repetitive spacers. Spoligotyping is performed by hybridization to synthetic spacer oligonucleotides, specific for each of the spacers, that are covalently bound to a membrane [110]. The PGRS locus consists of a 96 bp GC-rich consensus sequence that is present at multiple locations on the *M. tuberculosis* genome, whose distribution is assayed by Southern hybridization [243]. MLVA has been used extensively for pathogens with low genetic diversity, such as *Y. pestis*, *B. anthracis*, *M. tuberculosis*, and *Brucella* [31,78,115,132,189] and has gained considerable attention as a general method for the subspecies typing of relatively monomorphic species [50]. A web-based database containing tandem repeats from multiple bacterial genomes is now publicly available (http://minisatellites.u-psud.fr). Although the majority of tandem repeats is often not polymorphic [132,178], once more than one genome has been sequenced from a particular species, the database also allows the identification of polymorphic VNTRs [50].

Serology based on flagellins: Bacterial motility is based on the flagellum, an extracellular propeller constructed from 11 proto-filaments, each consisting of several thousand flagellin units. The N- and C-termini of flagellin are conserved in various bacteria, but the central portion is hypervariable [82]. Uniquely among the *Enterobacteriaceae, Salmonella* often possesses two distinct and alternative flagellins (H antigens) [155], encoded by two different genes, *fljB* and *fliC*, that are alternatively expressed due to flagellar phase variation [210]. The molecular mechanism mediating flagellar phase variation depends on a site-specific DNA inversion event that occurs with a rate of 10^{-3} to 10^{-5} per cell generation and which results in alternate expression of *fliC* and *fljB* [28]. Flagellins stimulate the host innate immune response strongly [92,174] and are subjected to strong selection. Flagellar phase variation has been postulated to play a role in *Salmonella* pathogenesis by allowing the bacteria to evade cellular immunity [70,100], but its exact role remains unclear [28]. The serological properties of flagellin proteins (and O-polysaccharide or O antigen) are the basis for serotyping with sera from experimental animals [155], which has been widely used for the diagnostic identification and epidemiology of *S. enterica*.

Methods based on avirulence genes: Avirulence (*avr*) genes render a plant pathogen unable to cause disease of hosts that express a corresponding resistance gene. Many *avr* genes code for virulence factors which are recognized by the product of the corresponding resistance gene to trigger defense mechanisms in the plant [17,148]. In many plant pathogenic bacteria (*Erwinia*, *Pseudomonas*, *Ralstonia*, *Xanthomonas*), most *avr* genes belong to the same family as *avrBs3* in *Xanthomonas vesicatoria*, which is recognized by *Bs3*, a pepper resistance gene to bacterial spot disease [27]. Genes of the *avrBs3* family are present in multiple copies in bacterial genomes, sometimes clustered with other pathogenicity-related loci in pathogenicity islands [101].

avrBs3-like genes have highly conserved N- and C-terminal regions plus a central region composed of 12.5–25.5 nearly identical tandem 102 bp repeats [253]. Variations in the

number, sequence, and order of the repeats in the central region are responsible for the high variability of host range, virulence, and aggressiveness among different bacteria [253]. *Avr* genes are prone to diversification because (i) mutations in the repeat core lead to new virulence characteristics [27], (ii) transposition of insertion elements can eliminate their avirulence function [126], (iii) intragenic or intergenic recombination between the repeats in the central region can result in new functionalities [48], (iv) these genes may have intrinsic Tn3-like transposition activity [75], (v) several *avr* genes are plasmid-borne or included in pathogenicity islands and can be transmitted to other strains or can be deleted [47].

Avr genes have the potential to reveal aspects of the population structure and evolutionary history of plant pathogenic bacteria that reflect their interactions with their hosts. Currently, attempts to control bacterial diseases consist primarily of introgressing resistance genes into cultivated plants, but this strategy is hindered when mutation or recombination generates novel *avr* genes that counteract the resistance genes or when bacteria containing specific combinations of *avr* genes are selected. For example, deciphering the short-term evolutionary history of *Xanthomonas oryzae* pv. *oryzae* by a combination of RFLP analysis of *avr* genes, rep-PCR, and IS-PCR data showed that the *Xa10* resistance gene in rice was easily overcome while the bacterial variants that could overcome the *Xa7* resistance gene were less aggressive and could not establish themselves under natural conditions [236]. Such data are valuable for the design of resistant plants by breeders, especially when matched with experiments that determine the evolutionary potential of the bacterial pathogenic populations [134,153].

28.2.1.3 Microarrays A microarray (or chip) is a solid substrate on which multiple nucleic acid targets are immobilized and can be used for hybridization with fluorescent probes, usually consisting of genomic DNA. The nucleic acid targets can consist of PCR products spanning each gene or part of a gene from a bacterial genome or 50–70mer oligonucleotides. Microarrays have been used for a variety of purposes with several bacterial species [33,254]. Microarrays have been used to detect differences in transcription in *E. coli* during phagocytosis [218] or in phagocytes due to the innate immune response [26]. Hence, microarrays have the potential to dramatically increase our understanding of the molecular basis of both sides of the host–pathogen interaction in infectious diseases [33]. Secondly, microarrays have been used for the identification of pathogens [142] and to analyze the composition of microbial communities [251]. Improvements in predicting the hybridization behavior of oligonucleotides will provide a high-throughput alternative for detecting and quantifying pathogens and may become a possibly powerful tool for microbial diagnostics [24]. Thirdly, microarrays allowed the assessment of genetic diversity based on the presence or absence of genes [19,94,186,198]. For instance, microarray analyses of *Salmonella* have revealed that the genomes of related *S. enterica* serovars differ by the presence or absence of 100s of genes [37,186,188], due largely to horizontal gene transfer and the integration of prophages [187].

28.2.2 Sequence Comparisons

Sequence variation is largely neutral or nearly neutral because most mutations that result in selective differences are deleterious [175] and are removed by purifying selection. However, genes involved in pathogenicity or whose products are immunogenic may contain sequence diversity due to diversifying selection mediated by interactions with the host immune system [213]. In contrast, the sequence diversity in housekeeping genes is probably largely neutral and accumulates with a relatively uniform molecular clock rate after diversification from a last common ancestor.

If all genetic variation were due to mutation, it would be easy to determine the patterns of phylogenetic descent within bacterial species. However, most of the sequence diversity between closely related *E. coli* [87], *N. meningitides* [68], or *Streptococcus pneumoniae* [69] results from recombination. Indeed, phylogenetic trees within many bacterial species are not statistically significant or are contradictory for different loci [66]. As a result it becomes important to compare the sequence diversity for multiple loci or even complete genomes in order to minimize the effects of recombination with single genes.

28.2.2.1 Multiple loci *MLST* [147]: MLST is a sequence-based replacement for MLEE that allows long-term epidemiology of bacterial species.

Approximately 500 bp gene fragments of multiple (typically seven) housekeeping genes are sequenced. Each unique sequence is assigned a distinct allele designation and each combination of alleles is assigned a distinct sequence type (ST) (Fig. 28.7). Relationships between bacteria are typically scored on the basis of Hamming distances between STs, that is, how

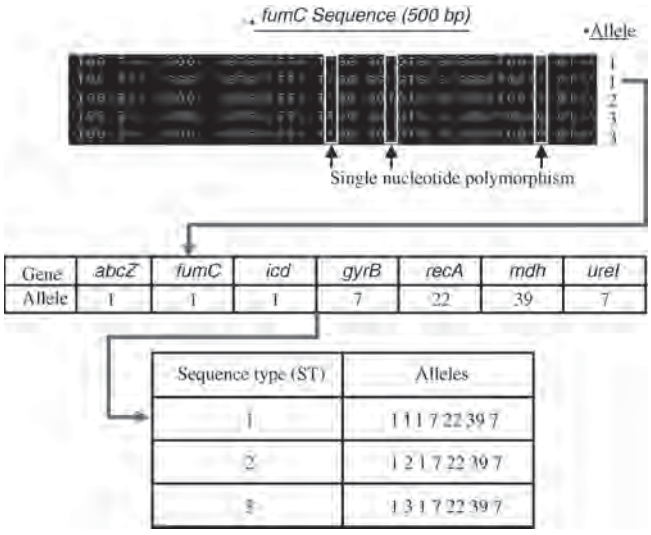

Fig. 28.7. Scoring of MLST data. Each unique sequence is assigned a distinct allele designation (for instance *fum*C allele 2) and each combination of alleles is assigned a distinct sequence type (e.g., ST1).

many alleles are identical, thus weighting changes due to mutations and recombination equally. Groups of STs that differ by no more than one to two alleles are referred to as clonal complexes [230]. Initially, clonal complexes can be recognized using the eBurst algorithm [67] but for larger datasets, manual curation is still necessary. Global MLST of 20 species can be found at http://www.mlst.net. As of September 2004, the largest MLST databases are *Neisseria* (6006 isolates; 4153 STs), *Campylobacter* (2352 isolates in 1015 STs), and *S. pneumoniae* (2269 isolates in 1384 STs). MLST has become the gold standard for the recognition of genetically related bacteria and long-term epidemiology of bacterial pathogens [52,58,65,80,96,108,119,125,147,156,199,220].

MLST as described above is not suitable for all bacterial species. Some pathogenic bacteria are so young that too little genetic diversity exists for differentiation by MLST [8,119,217]. Others, such as *H. pylori*, cannot be assigned to STs because almost all isolates have unique genotypes [63]. However, sequence-based data from housekeeping genes can be used to recognize populations in *H. pylori* with Bayesian analyses [62,63] and have even been used as a marker for differentiating their human hosts [249]. *H. pylori* are largely transmitted in families, after which they persist in single hosts for decades. As a result, their population biology mimics that of their hosts, and reflects ancient human migrations, such as the prehistoric colonizations of Polynesia and the Americas, the Neolithic introduction of farming to Europe, the Bantu expansion within Africa, and the slave trade [63].

Single nucleotide polymorphisms (SNPs): SNPs represent point mutations within populations that have not (yet) been removed by drift. Point mutations have lower mutation rates than minisatellites or other contingency loci. Polymorphisms within coding sequences that result in amino acid changes are referred to as nonsynonymous while other SNPs are designated synonymous or silent. SNP genotyping has been widely used with human genomes for the fine mapping of disease loci and for identifying candidate genes [38]. These studies have resulted in huge SNP datasets for the human genome and for model organisms such as *Drosophila melanogaster*, *Mus musculus*, and *Arabidopsis thaliana* [20,39,97,135,224,242].

Single-strand conformational polymorphism (SSCP) has been used to detect SNPs and larger sequence polymorphisms in fragments of up to 1000 bp for over a decade [179]. SSCP is based on mobility changes during electrophoresis in acrylamide gels that often accompany changes in single-stranded DNA conformation due to altered intramolecular base pairing. SSCP has now largely been replaced by newer technologies that are more suitable for higher throughput and can be more cost-efficient, such as SNaPshot and SNPlex (http://www.appliedbiosystems.com) and dHPLC (denaturing high performance liquid chromatography) [42]. Very high throughput SNP testing can also be performed by mass spectrometry and array-based resequencing [38,117,158].

SNP analysis has not yet been used extensively for pathogenic bacteria. SNPs based on genomic sequences have been used to investigate the phylogenetic structures of *M. tuberculosis* [86], *B. anthracis* [184], and *Y. pestis* [6]. In each case, the SNPs were based on only a few genomes and the shapes of the resulting trees are crucially dependent on whether the genomes represent the maximum diversity of the species under investigation [184], which is unfortunately rarely the case. Very distinct bacterial strains can yield only moderately different SNP patterns if the genome sequences available represent only a fraction of the genetic diversity within the species, as was the case for *M. tuberculosis* [86]. Therefore, genomes used to define SNPs should represent the maximum diversity of a species as defined by other independent methods such as MLVA [6,184].

Once reliable SNP-based genotyping has been established for a species, it is possible to choose a smaller subset of canonical SNPs to rapidly assign isolates to groups or populations [116]. Similarly, SNPs of importance for pathogenesis (pathoadaptive SNPs) might be valuable tools for the dissection of host–pathogen interactions. Examples of pathoadaptive SNPs have been described for the FimH and Dr adhesins in *E. coli* [35,215] and *S. enterica* Typhimurium (only FimH) [245].

28.2.2.2 Whole genome comparison The first bacterial genome was published in 1995 [72]. Twelve years later, 357 complete bacterial genome sequences from 264 bacterial and archaeal species have been made publicly available (http://www.cbs.dtu.dk/services/GenomeAtlas/). These sequences provide new insights on the molecular basis of pathogenesis, host range, evolution, environmental survival, and phenotypic differences. There are clear trends in gene content as the genome size increases [124]. Compared to medium and small size genomes, large genomes are disproportionately rich in loci involved in regulation and secondary metabolism and depleted in loci needed for protein translation, DNA replication, cell division, and nucleotide metabolism genes. Horizontally transferred genes and their possible donors have been identified in bacterial genomes by Bayesian inference [166]. Fourteen percent of coding sequences had been introduced by horizontal transfer. The putative functions of most of these genes belong to three functional categories: cell surface, DNA binding, and pathogenicity related [166]. These latter studies are the first comparative analyses of numerous complete genomes and pave the road for new insights in evolution and adaptation.

Genome sequences are also providing information on diversity among closely related organisms and multiple genome sequences are now available for at least 30 bacterial species or genera [64]. As a result, it has become increasingly clear that host adaptation is often accompanied by genome decay [12,159,182,183], although this is not universally true [16,170].

Bacterial genomes seem to be composed of two distinct parts: the "core" genome and "accessory" genes [129]. The core genome consists of genes that are ubiquitous within all strains of a species, partly because they are necessary for growth, while accessory genes are involved in supplementary

biochemical pathways, transposition, and fitness. Accessory genes are often grouped in DNA islands that are variably present in different strains [10,127], and are thought to have often been introduced by horizontal gene transfer or prophage integration. As the numbers of sequenced genomes within species increases, there will be qualitative jumps in our understanding of microbial diversity.

28.3 APPLICATIONS OF MOLECULAR METHODS

The ability to distinguish between bacteria is essential to a variety of microbiological approaches, including taxonomy, phylogenetics, population genetics, epidemiology, and diagnostics [231]. A plethora of techniques has been described for this goal, some of only limited and transient importance [1], and a primary question has become the choice of appropriate types of markers and analytical algorithms for data analysis [221]. Markers with different clock rates are needed to differentiate within species that have accumulated considerable diversity over millions of years. Conversely, only markers with high mutation rates are appropriate for recently evolved species with little diversity (Fig. 28.8).

Similarly, the clock rates that are most appropriate for reliable subdivision into bacterial populations differ from those best suited for discriminating between individual strains. Finally, the choice of technology should depend on the sample size and the desired life span for epidemiological comparisons.

28.3.1 Clock Rates of Different Markers

As described above, clock rates for different types of markers differ by orders of magnitude, varying from very fast for VNTRs and other contingency markers to very slow for SNPs. In addition, the ratio between the clock rates for housekeeping genes to ribosomal RNA can vary by orders of magnitude between different bacterial species [172]. Unfortunately, very few clock rates have been estimated for the different markers described here and it is difficult to predict the time scale over which they are most appropriate. IS*6100*-RFLP patterns of serial isolates from single patients change with a half-life of 3 [45] to 9 [244] years, largely due to changes that occur within the first 20 days after diagnosis [57] within a small number of patients [169]. Based on these results, IS*6100*-RFLP seems to be suitable for detecting recent transmission clusters but less suitable for long-term epidemiology. MLVA patterns based on 43 VNTRs in *Y. pestis* changed with an average frequency of 6% per transmission between prairie dogs in a local outbreak [78]. Similarly, variability was observed among seven VNTRs linked to virulence among related serial isolates of *Haemophilus influenzae* from lung infections of patients suffering from cystic fibrosis [194]. Thus, VNTRs may be ideal for detecting genetic variability among related organisms over short time periods. Multiple changes in the immunogenic TbpB protein due to horizontal gene transfer from commensal neisseriae were observed during a single epidemic [137] and during global spread [255] of serogroup A *N. meningitidis* whereas MLST

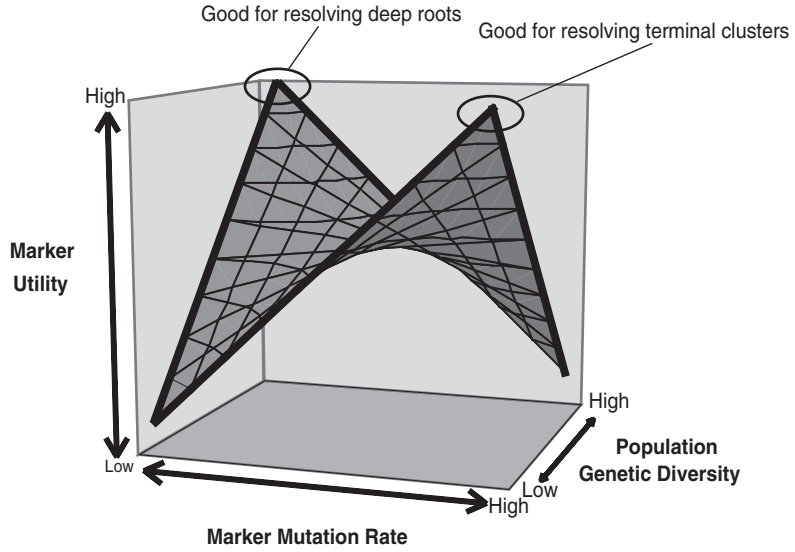

Fig. 28.8. The utility of a genetic marker for determining phylogenetic relationships within a given population is a function of (i) the mutation rate of the marker and (ii) the overall genetic diversity of the examined population. When genetic diversity of the population is low (terminal cluster), only markers with high mutation rates, such as single nucleotide repeats, will be able to differentiate among individuals in the population. Conversely, when genetic diversity of the population is high (e.g., an older and deeper phylogenetic group), only markers with low mutation rates, such as SNPs, will yield accurate phylogenetic patterns, as information obtained from markers with higher mutation rates is likely to be distorted by homoplasy (from Keim et al. [116], with permission).

patterns were largely constant. These observations support the concept that MLST is primarily suited for long-term epidemiology. Finally, sequence differences due primarily to recombination were detected in 6/22 pairs of serial isolates of *H. pylori* that were isolated on average 1.8 years apart [61]. Based on these data, it was calculated that strains of *H. pylori* import 25 kb of their genome per year from other *H. pylori* that co-colonize the stomach.

High clock rates, such as those described above, may present difficulties for the analysis of entire species or populations because they result in homoplasies and high noise levels. Therefore, populations may best be recognized with markers with slower clock rates, even if they are not as discriminatory. These include canonical SNPs for *B. anthracis* [116], spoligotyping for the *M. tuberculosis* complex [89,212], synonymous SNPs for *Y. pestis* [6], multilocus sequence assignments by Bayesian analysis for *H. pylori* [63], and classical MLST for numerous bacterial species [230]. Molecular epidemiology needs to combine two partially contradictory approaches, classification and typing, in order to yield the maximal information about bacterial disease. Classification should be based on conservative properties that index the phylogenetic descent of the organisms, whereas typing should use molecular markers with high mutation rates, including variable virulence determinants, to enable fine discrimination of individual isolates.

28.3.2 Geographical Considerations

Most publications on molecular typing concentrate on samples from local or at most national sources. Such analyses are directly relevant to the epidemiological mandate of many sources of funding and are easier to organize than are analyses of global samples. Yet, bacteria can sweep around the world in months or years [130,255], as can viruses. MLST can provide notice of transmission between countries or continents of an ST that was previously absent. Geographical differentiation has been described for a number of species, including *H. influenzae* [164], *Actinobacillus actinomycetemcomitans* [91], the *M. tuberculosis* complex [79,89,95], and *H. pylori* [63]. Our global overview of these organisms is currently most extensive for *H. pylori* but even for that species, we still lack information for large parts of the world. It seems clear that future work on this area has considerable promise but will also require long-term planning regarding global surveying with durable methodologies.

28.3.2.1 Long-term epidemiology Methodologies that are suitable for long-term epidemiology should be capable of typing all isolates with universal markers, must be portable between laboratories, and allow ready international access. Portability depends on standardized technologies and high reproducibility between laboratories. Standardization on a global scale has been implemented for IS*6100*-RFLP of *M. tuberculosis* [234] and in the United States for PFGE for bacterial food-borne pathogens [223]. However, interlaboratory comparisons suggest that IS*6100*-RFLP patterns are not sufficiently reproducible for complex patterns with large number of bands [30]. Furthermore, it has apparently not been possible to establish reliable global databases based on these methods. It seems likely that long-term analyses will increasingly be based on DNA sequencing or SNPs because they yield reproducible results in different laboratories through the use of commercially standardized protocols. MLST has become the gold standard for *N. meningitidis* and multiple other species of pathogens (http://www.mlst.net), particularly because curated global databases allow data entry and retrieval via the internet and facilitate the pooling of resources by the global scientific community for clinical diagnosis, epidemiological monitoring, and population studies. Recent technological developments have increased the ease and substantially reduced the expense of nucleotide sequence determination. Costs as low as €15 per isolate are now achievable, and further cost reductions are likely in the near future [230].

A consensus has not yet been reached for standardized methods for long-term epidemiology of largely monomorphic species such as *M. tuberculosis* and *Y. pestis*. Considerable attention has been recently devoted to VNTRs [50,116], but it remains to be seen whether globally accessible databases based on VNTRs will be established and gain acceptance. Although only few bacterial studies have been based on SNPs, current advances in technology driven by eukaryotic questions [158] may well turn SNP analysis into the method of choice for many species.

28.3.2.2 Local epidemiology Many of the techniques described above are suitable to detect relationships among isolates collected locally over short time intervals and can reveal the dynamics of nosocomial infections or allow monitoring of pathogens during local outbreaks. A variety of criteria should be considered, including whether the methods are capable of being performed with high throughput and how quickly they can supply decision makers with data. Additional requirements are how widely applicable the methods are, how well they can discriminate between unrelated strains, ease of performance and interpretation, and cost.

PCR-based methods, such as AFLP, enable the diagnosis of numerous samples, do not require cultivation, and can provide data sufficiently rapidly for the surveillance of nosocomial disease, local outbreaks, and forensic applications. Real-time PCR systems have the potential of reducing diagnostic turnaround times to several hours [239]. Antibiotic resistance in nosocomial organisms, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci, can be determined routinely by PCR-based methods [239]. RAPD and PFGE are also well suited for small-scale studies, such as the investigation of a hospital or local outbreak, in which a limited number of samples is collected within a narrow time frame [41,76]. Such analyses might help with the clinical management of infected patients because they could distinguish new infection from relapse, determine the significance of colonization with potential pathogens, assess the

capacity of isolates to spread and cause serious illness, and link changes in microbial resistance with treatment [99].

28.3.3 Hierarchical and Nested Approach

The combination of methods of different resolution on subsets of isolates can be more productive than the use of each method alone on larger numbers of isolates. For example, after using local epidemiology to group related strains, representatives of each group can be tested by methods more suitable for long-term epidemiology [76]. Alternatively, a nested top-down approach called progressive hierarchical resolving assays using nucleic acids (PHRANA) has been suggested for *B. anthracis* [116] in which organisms are first grouped by canonical SNPs, then typed by VNTRs and finally, very closely related strains are differentiated by size-variation in homopolymeric stretches (Fig. 28.9).

Within this approach, the group of organisms that is subdivided by each method possesses the appropriate range of variability for the particular method used. PHRANA might be applicable to many other species once markers with variable diversity values have been defined.

28.3.4 Population Genetics

The availability of large databases provides data for population genetic studies, which in turn can help explain patterns of strain diversity within the databases. Antibiotic resistance may be the most important evolutionary change that microbiologists have been able to study in real time [150], resulting in mathematical models that may help physicians decide on optimal strategies for antibiotic usage [21,138]. MLST data have been used to estimate recombination and mutation rates [61,66], and to estimate the time since the last common ancestor for *Y. pestis* [8], *Shigella* [190], *E. coli* O157:H7 [193], and *S. enterica* Typhi [119]. Finally, Bayesian statistics have been used to subdivide *H. pylori* into multiple populations and subpopulations (Fig. 28.10) on the basis of sequence data [62].

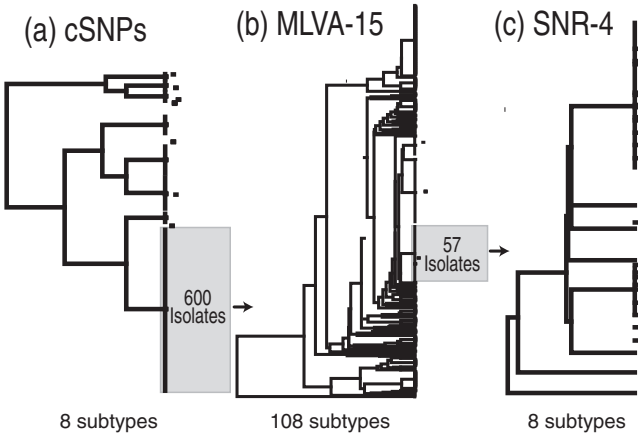

Fig. 28.9. PHRANA. (a) Eight canonical SNPs separated 1067 *B. anthracis* isolates into eight major phylogenetic groups; (b) MLVA-15 analysis of the 600 isolates in the largest cluster identified 108 unique types; (c) SNR-4 analysis of one of the MLVA types containing 57 isolates yielded eight unique PHRANA genotypes (from Keim et al. [116], with permission).

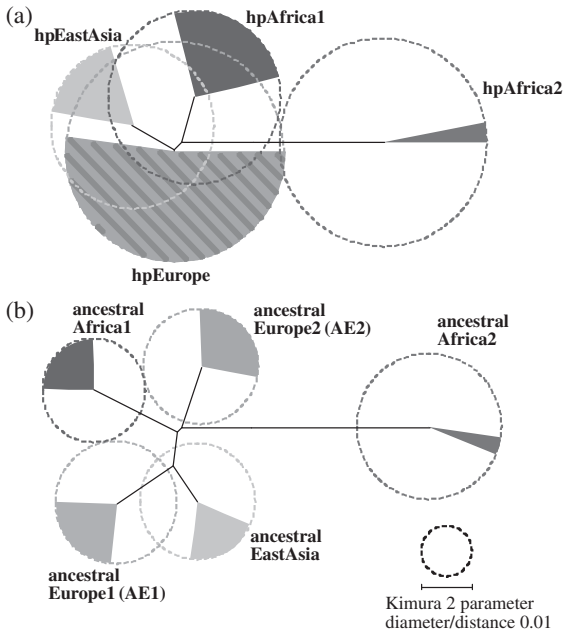

Fig. 28.10. Relationships between modern populations (a), modern subpopulations (b), and ancestral populations (c) of *H. pylori*. The black lines show neighbor-joining population trees as measured by δ, the net nucleotide distance between populations. The circle diameters indicate their genetic diversity, measured as the average genetic distance between random pairs of individuals. The larger circles in (a) versus (c) reflect the effects of admixture between ancestral populations. Filled arcs reflect the number of isolates (a and b) or polymorphic nucleotides (c) in each population. Color coding is consistent in different parts of the figure, except for modern hpEurope, which is an admixture between the ancestral AE1 and AE2 populations. Scales are at lower right (from Falush et al. [63]). Permission has been obtained. See color plates.

These statictics also allowed a reconstruction of the genetic diversity of the ancestral populations within *H. pylori*, prior to the extensive recombination, which is present in modern populations (Fig. 28.10).

28.4 CONCLUSIONS

Future studies of population structure and the evolution of bacterial species will require DNA sequences from multiple loci and large databases, such as are provided by MLST and SNP-typing. As these techniques become cheaper and faster, they will also become essential for typing and long-term epidemiology. This scenario demands independent curation in order to ensure that combined data from multiple laboratories are reliable. A further requirement is that sampling strategies reflect the biology of the particular pathogen and include representative material from the developing world. Cholera and tuberculosis provide prime examples where global collaboration on international problems is essential.

For some bacterial species, the primary current problem is a lack of knowledge about population structure and the availability of adequate statistical methods. Understanding population structure demands knowledge about epidemic processes and pathogen evolution that are still lacking. Many important parameters for bacterial populations need to be calculated, including size, growth rate, and generation time. The relative importance of diverse evolutionary processes also needs quantification, including mutation, homologous recombination, and horizontal genetic exchange. In addition to these classical parameters, we are largely ignorant about the details of microevolution of bacterial populations in nature.

Knowing population parameters would aid typing. For example, determining clock rates for different types of genes could facilitate the choice of appropriate genes for different questions. Similarly, genomic approaches such as microarray will help distinguish between neutral (core) and variable (accessory) genes. Neutral genes are more appropriate for analyses of the demographic and evolutionary history of bacterial populations whereas variable genes could shed light on the processes of adaptation and speciation. Increased knowledge about these processes will in turn facilitate tracking the spread of pathogens and the identification of vaccine-escape or antibiotic-resistant variants. Finally, such knowledge will facilitate clinical management of infected patients based on collaborations between molecular microbiologists and clinical microbiologists.

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CHAPTER 29

Exploring Genetic Relatedness, Patterns of Evolutionary Descent, and the Population Genetics of Bacterial Pathogens Using Multilocus Sequence Typing

Brian G. Spratt, William P. Hanage, and Christophe Fraser

Department of Infectious Disease Epidemiology, Imperial College London, St. Mary's Hospital Campus, London W2 1PG, UK

29.1 INTRODUCTION

The ability to discriminate between different isolates of pathogens and parasites is central to understanding modes of disease transmission, identifying outbreaks of disease, tracking the spread of drug-resistant strains, and monitoring the impact of intervention measures. Many of the widely used molecular typing methods characterize isolates of pathogens by comparing patterns of DNA fragments generated by digesting the chromosome with restriction endonucleases, and therefore index genetic variation that is difficult to compare between laboratories. These methods were developed for investigating local outbreaks in hospitals, or the community, where the key issue is whether isolates from different individuals are indistinguishable in genotype, implying cross-infection or an outbreak, with less interest in how the isolates are related to those recovered in other hospitals or communities. In recent years, there has been increasing interest in the spread of pathogens within and between countries and, consequently, in developing methods of characterizing pathogen isolates that use digital data, so that isolates studied in different laboratories may readily be compared using the Internet. There is also an increased awareness of the relevance of population and evolutionary biology to studies of infectious disease and of the advantages of characterizing pathogen isolates using genetic variation that is amenable to population genetic analysis.

There are a number of typing methods that produce digital data. The most venerable of these is multilocus enzyme electrophoresis (MLEE), which assigns alleles at multiple metabolic (housekeeping) loci indirectly from the electrophoretic mobility of their gene products on starch gels [30]. MLEE characterizes each isolate by a string of integers that corresponds to the allele numbers assigned to each of the different mobility variants at each locus. MLEE, therefore, produces digital data (an allelic profile), but the digital data are imprecise as they are based on measures of enzyme mobility in an electric field, making it difficult to compare results between laboratories. MLEE has been superseded by multilocus sequence typing (MLST), which uses the same principles as MLEE, but assigns alleles directly from the sequences of internal fragments of multiple (typically seven) housekeeping genes [24]. Thus, the data produced by MLST are both digital and precise, allowing an easy and unambiguous comparison of isolates between laboratories. Sequence data are also ideal for strain characterization via the Internet, as the sequence of each housekeeping gene fragment from a new isolate can be compared with those of all known alleles at the locus, using allele databases maintained at the MLST web site for that species, and can be assigned as known or novel alleles. Similarly, the allele numbers assigned at each of the seven loci (the allelic profile) can be compared with all of those in the strain database at the MLST web site, and an isolate

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can be assigned as having a known allelic profile, or a novel allelic profile, and entered in the strain database. Each different multilocus genotype (allelic profile) is assigned as a sequence type (ST), which provides a convenient descriptor for the strain. The sequence variation within the loci used in MLST is considered to be selectively neutral, or nearly so, and thus provides a far more reliable indicator of the relationships between strains than would be obtained by using sequence variation at loci that are subject to diversifying selection [24].

MLST is providing a valuable tool for molecular epidemiological studies of bacterial pathogens [5,7,8,9,17,19,20,22, 27,28,36] and is also being used to explore diversity and geographic substructure in environmental species [37]. The method can be applied to clinical samples in the absence of a culturable isolate (or to non-culturable species), as long as the fragments can be amplified by PCR. For invasive bacterial infections this is straightforward, as samples from non-sterile sites will almost invariably include only a single isolate of the pathogen, but is problematic for samples from infections or body sites where multiple isolates may be present.

MLST has been applied also to haploid and diploid fungal pathogens (e.g., *Candida albicans* and *C. glabrata* [3,6,34]). Complications arise with diploid organisms as some nucleotide sites will be heterozygous, but these can be readily identified and assigned standard ambiguity codes defined by the International Union of Pure and Applied Chemistry (IUPAC), allowing diploid STs to be assigned. Clusters of identical or very similar strains can then be identified and compared to others via the Internet [4].

The use of MLST for molecular epidemiology has been discussed elsewhere [36], and here we focus on how MLST data can be used to investigate the relatedness between isolates and, particularly, to explore recent evolutionary events. We also briefly discuss ways in which MLST data can be used to explore aspects of the population genetics of bacteria.

29.2 BACTERIAL POPULATION STRUCTURE AND MLST

MLST was introduced primarily to allow isolates of bacterial pathogens to be unambiguously characterized via the Internet, as an aid to epidemiological studies, rather than as a tool to analyze bacterial population structures [24]. However, the method is a development of MLEE, which has provided many of the basic principles of bacterial population genetics [30], and both methods assign alleles at multiple housekeeping loci and thereby define the multilocus genotypes of isolates, and such data are amenable to population genetic analysis. MLST has an additional advantage over MLEE as the sequence data can be analyzed as well as the frequencies of alleles and multilocus genotypes within a population.

It has been evident for many years that bacteria are not strictly clonal [12,25]. Recombination occurs in most bacteria, although it is highly localized, resulting in the replacement of a small region of a recipient chromosome with the corresponding region from a donor strain. Natural transformation provides the mechanism for these sexual exchanges in some species, and in non-transformable species, it is believed that phage-mediated transduction is the dominant mechanism. The average size and the size distribution of the recombinational replacements are not well defined, but are believed to be a few hundred base pairs to a few kilobases in transformable species, and probably somewhat larger where transduction is the mechanism [12]. The frequency of homologous recombinational replacement appears to vary widely between species, and may vary significantly within a species, as a result of differences in the efficiency of genetic exchange. The impact of recombination will also depend on the extent to which different strains of a species meet each other in nature, as even frequent recombination will have no genetic consequences if it occurs between identical isolates.

The extent of recombination is important as it can impact on the epidemiological questions that can be addressed and whether MLST can provide a useful approach to molecular typing [31]. If rates of localized recombination are very high the rate of genetic diversification of strains is likely to be rapid (as long as different strains meet each other) and, if there is sufficient genetic variation within the population, isolates from epidemiologically unlinked individuals will have different multilocus genotypes. Rapid diversification of genotypes will make clones unstable, and perhaps not even evident in the population, which precludes studies of the spread of strains within or between communities. MLST may therefore provide a highly discriminatory typing method that can be used to analyze outbreaks, but the epidemiological questions that can be addressed in such species are likely to be limited. A good example is *Helicobacter pylori* where rapid diversification prevents the identification of clones within communities [32], although MLST may still be useful for addressing local epidemiological questions, such as understanding pathways of transmission within families, or distinguishing whether a relapse of disease after antibiotic treatment is due to a failure to clear the infection or to reinfection with a different strain.

Conversely, an absence of recombination, or very low rates of recombination, will result in the very slow divergence of STs by the gradual accumulation of point mutations in the housekeeping genes and a highly clonal population structure. MLST provides an appropriate way of defining genotypes and of recognizing important lineages in such populations. However, MLST may be of limited utility for some epidemiological purposes as there may be too little variation at housekeeping loci within the important disease causing lineages.

A second aspect of pathogen biology that impacts on the utility of MLST as a tool for molecular epidemiological studies is the genetic diversity within a pathogen. MLST requires sufficient numbers of alleles at each locus to provide a high level of discrimination between isolates. In some pathogens there is almost no sequence variation at housekeeping loci and MLST does not provide adequate discrimination between strains. In many cases this lack of genetic variation is a consequence of species status having been given to a single clone that causes a

distinctive disease (e.g., *Yersinia pestis* and plague [1], or *Burkholderia mallei* and glanders [17]). Whatever the reason, a lack of variation at housekeeping loci among the isolates causing a disease makes MLST inappropriate and other types of genetic variation must be sought. Several diseases are now known to be caused by these monoclonal or oligoclonal pathogens, including tuberculosis, typhoid fever, plague, anthrax, and glanders [31]. In some other pathogens there is more genetic variation within housekeeping loci, but still not enough to provide a highly discriminatory MLST scheme (e.g., *Neisseria gonorrhoeae*), but in many important pathogens there is sufficient variation for MLST to be a useful approach.

29.2.1 Displaying Relationships Between Isolates

The digital nature of the data produced by MLST makes it very simple to interrogate the appropriate MLST web site to establish whether an isolate corresponds to a known ST, or is novel, and to identify isolates in the database that have some defined level of similarity in their allelic profile to the query isolate. Displaying the relatedness of all isolates within an MLST database is challenging as several of these include thousands of isolates. The relatedness of isolates is usually determined by constructing a dendrogram based on the matrix of pairwise differences between the allelic profiles of the isolates. This provides a convenient visual representation of the extent of clustering in the population, and identifies those isolates that are indistinguishable (clones) or very similar in allelic profile (clonal complexes). The problem with this approach (besides the very large numbers of isolates) is that isolates descended from a common ancestor will soon, on evolutionary time scales, accumulate at least one nucleotide change at each of the seven MLST loci and will then appear to be totally unrelated in genotype. The dendrogram, therefore, cannot be used to explore the relatedness of the more distantly related isolates in the population (Fig. 29.1a).

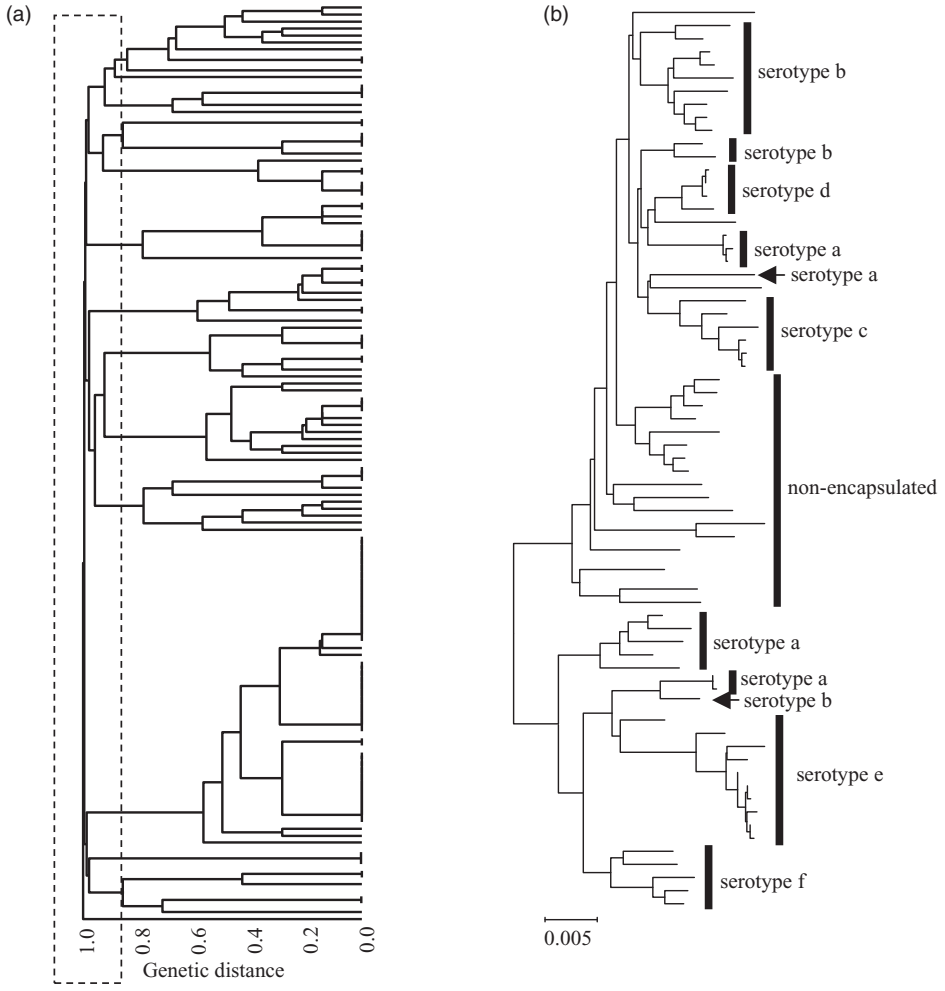

Fig. 29.1. Relatedness among *H. influenzae* inferred from differences in allelic profiles and concatenated sequences of MLST loci. (a) UPGMA dendrogram constructed from the pairwise differences in the allelic profiles of all isolates of the first 68 STs in the *H. influenzae* MLST database. Nodes within the dotted rectangle link lineages that differ at six or all seven of the MLST loci. (b) The sequences of the seven MLST loci of the first 68 STs were concatenated and a minimum evolution tree was constructed. The serotypes of the isolates in some clusters of related STs are shown (modified from [27]).

This lack of temporal depth in trees based on differences in allelic profiles can be circumvented by using the sequence data to construct trees. The sequences of the seven loci can be joined end-to-end (concatenated), maintaining the correct reading frame, and the concatenated sequences can be used to construct a tree (Fig. 29.1b). This approach appears attractive, but whether the deeper relationships in a tree constructed using concatenated sequences are a reasonable representation of the divergence of these lineages will depend on the impact of recombination [14]. In some bacterial species, the extent of recombination may be sufficiently low that the tree adequately represents the intraspecies phylogeny [14]. However, in many species, recombination over the long term is probably frequent enough to result in a loss of phylogenetic signal, so that the relatedness of divergent lineages cannot reliably be ascertained [13]. Even with relatively closely related isolates, a tree based on concatenated sequences may give results that are less reliable than those obtained using the differences in allelic profiles. Diversification of a pair of identical isolates will lead to descendents that differ at one, two, three, and so on loci and, with a tree based on differences in allelic profiles, is uninfluenced by whether the alleles change by recombination or point mutation. However, if concatenated sequences are used, particularly with diverse species, each allelic change may introduce many nucleotide changes if recombination is involved, or only one if the allele changes by a point mutation. Descendents that differ at three loci could therefore differ at three nucleotide sites, or at thirty, and may appear to be very closely related, or relatively distantly related, on a tree based on sequence differences. The relatedness of recently diverged isolates, and the identification of clonal complexes, in relatively recombinogenic species may therefore be represented better by a tree based on differences in allelic profiles than one based on differences in nucleotide sequences. Recent approaches to identify clonal complexes and to explore the relatedness of isolates within clonal complexes are discussed further in Section 29.2.2.

A number of methods have been introduced that attempt to show the relationships among isolates of a species without assuming that evolution has occurred in a tree-like manner. The first of those to be widely used was split decomposition analysis (the Splits Tree program; http://bibiserv.techfak.uni-bielefeld.de/splits), which displays the implied relationships between isolates, but shows alternative pathways where there is conflict in the data [2]. These alternative pathways (splits) are generally assumed to be due to recombinational replacements which result in the same polymorphisms being present in otherwise divergent sequences. Splits Tree should produce output that is similar to a conventional phylogenetic tree for bacteria where there is little recombination, but, in a recombining species, a net-like diagram (a splits graph) is produced showing the conflicts in the data that support multiple plausible evolutionary pathways between the sequences. When applied to the concatenated sequences from the MLST loci this approach can give a visual indication of the extent of conflict in the data, and thus of the likely extent of recombination. Splits Tree can also be applied to allelic profiles where it can show the relationships between very similar isolates, and alternative conflicting pathways between more distantly related isolates, although the eBURST method (see Section 29.2.2.1) provides a much more informative procedure for exploring the relatedness between similar isolates.

29.2.2 Defining Clonal Complexes, Clonal Ancestry and Patterns of Descent

Dendrograms based on differences in allelic profiles are a useful tool for visualizing the clusters of related isolates, and concatenated sequences can be used to explore the deeper relationships between the clusters in species where rates of recombination are low. In many bacterial species a history of repeated recombinational replacements may preclude the determination of the true genetic relationships among divergent lineages, but it may still be possible to explore recent ancestry. Much of the interest in bacterial pathogen populations relates to relatively recent events and, at least for epidemiology and public health, there may be little interest in knowing the phylogenetic relationships between distantly related lineages. Thus, the emergence and spread of antibiotic-resistant strains, or virulent strains, or changes in a pathogen population imposed by a vaccine, are recent events that could be explored if we had methods to determine ancestry and patterns of descent among sets of similar isolates.

29.2.2.1 eBURST The relationships between closely related bacterial isolates are poorly represented by a tree. A tree indicates the clustering of the isolates with similar genotypes, but it gives no information about the most plausible evolutionary pathways between very similar isolates. Information about ancestry, and the pathways of descent among closely related isolates, is present in MLST data and a new method, eBURST, is designed to extract and display this information [15]. eBURST makes no attempt to discern the relationships between divergent isolates, but focuses on identifying groups of closely related isolates within populations, and on identifying the likely ancestral or founder genotype of each group, and the pathways of evolutionary descent from that founder to all of the other members of the group. The eBURST approach extracts information about ancestry and pathways of descent as it incorporates a simple model of the way in which bacterial clones emerge and diversify. According to this model, genotypes occasionally increase in frequency in the population (under selection or drift), and gradually diversify to produce descendents that are increasingly variable in genotype. In the context of MLST, the isolates of such emerging clones will initially be indistinguishable in allelic profile (i.e., the same ST), but the accumulation of mutations or recombinational replacements will eventually result in the appearance of variants that differ at only one of the seven MLST loci (single-locus variants; SLVs). A young clonal complex will therefore consist of many isolates of the ancestral or founder ST with smaller numbers of isolates of its multiple descendent SLVs. The founder ST can therefore

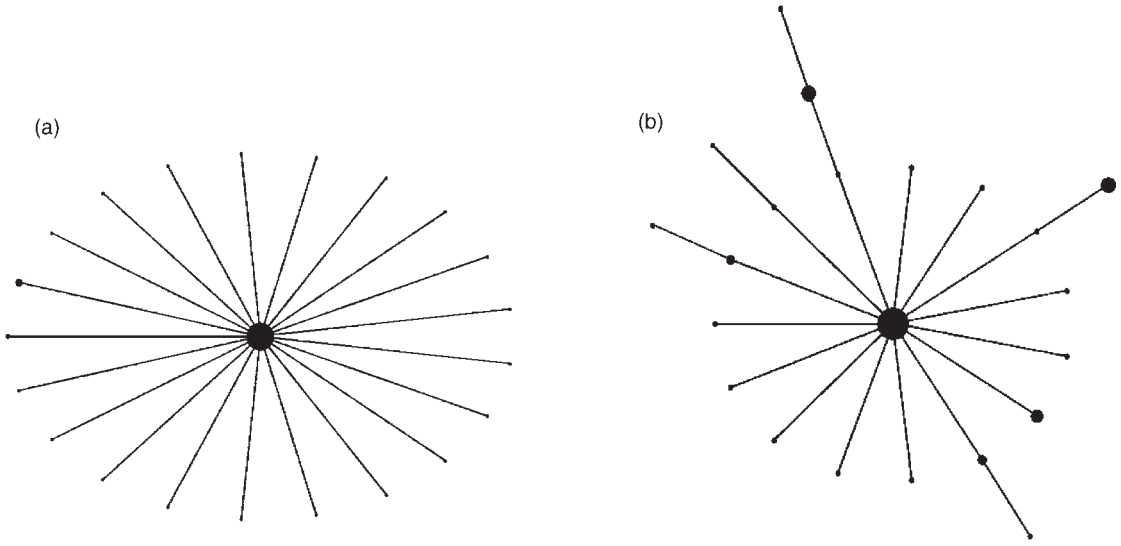

Fig. 29.2. Displaying relatedness within clonal complexes using eBURST. The eBURST program was used to divide all isolates in the *S. pneumoniae* and *H. influenzae* MLST databases into groups of related isolates using the stringent default group definition. (a) ST81 is assigned as the founder of the *S. pneumoniae* clonal complex that correponds to a major multidrug-resistant clone. This clone is young, and the majority of isolates have the founding genotype (ST81), but it has diversified to form a number of SLVs. (b) The major clonal complex of *H. influenzae* serotype b (hib) is more diversified and some of the SLVs of the founder (ST6) have diversified further to produce DLVs and in one case a TLV. The size of the circles that correspond to STs represents the prevalence of the ST in the input data. ST numbers are not shown.

be recognized as the ST within a clonal complex that has the greatest number of SLVs. It is also likely that the founder ST of a young clonal complex will be the most prevalent ST in a well-sampled population and this expectation is generally upheld. In older clonal complexes, further diversification will have occurred, to produce double-locus and triple-locus variants of the founder ST, and some of these descendents of the founder may themselves have become prevalent and have diversified to form their own sets of descendent SLVs [15].

The eBURST program takes as input the ST and allelic profile of each isolate in the population and identifies nonoverlapping groups of STs that share some user-defined level of similarity in their allelic profiles. The default definition of these eBURST groups is that each ST assigned to the same group has at least six of its seven alleles in common with at least one other ST in the group. Using this stringent definition, the STs assigned to an eBURST group are defined as a clonal complex, with the assumption that they are all descended from the same founder ST. The founder ST of each eBURST group is then predicted and the extent of statistical support for the founder is provided by a bootstrap resampling procedure [15]. The pattern of descent from the predicted founder ST to all other STs in the eBURST group is then inferred and the results are displayed as an eBURST diagram. The eBURST diagram only links (joins) STs that differ at a single locus and, using the default definition, all STs are linked because each must be a SLV of at least one ST in the group. A simple eBURST group typically includes the founder ST, which is placed centrally, surrounded by a number of linked descendent SLVs, some of which may have diversified further to produce double-locus variants (DLVs) or triple-locus variants (TLVs) of the founder ST (Fig. 29.2). The sizes of the circles that represent individual STs in eBURST diagrams indicate their prevalence in the input dataset. The eBURST program can be run from within the www.mlst.net web site, and the program links directly to the appropriate species database so that the isolates of any ST selected within an eBURST diagram, and the isolates of all its SLVs, can be extracted from the database and viewed.

The eBURST diagram produces an hypothesis about ancestry and patterns of descent that can be explored further by mapping on additional phenotypic or genotypic data [10]. A good example of the ability of eBURST to produce an hypothesis about ancestry is shown by an analysis of the large lineage 3 clonal complex of *N. meningitidis,* which was originally defined by MLEE as a cluster of related strains associated with meningococcal disease in the Netherlands [29]. Figure 29.3(a) shows the lineage 3 part of a large tree constructed using differences in the allelic profiles of each meningococcal ST in the public *Neisseria* MLST database (http://pubmlst.org/neisseria/). The tree identifies two large clusters of STs that appear to be related at a genetic distance of 0.57, but provides no further useful information about how this clonal complex arose. The eBURST diagram, obtained using the stringent default definition of an eBURST group, links most of these lineage 3 STs into a single

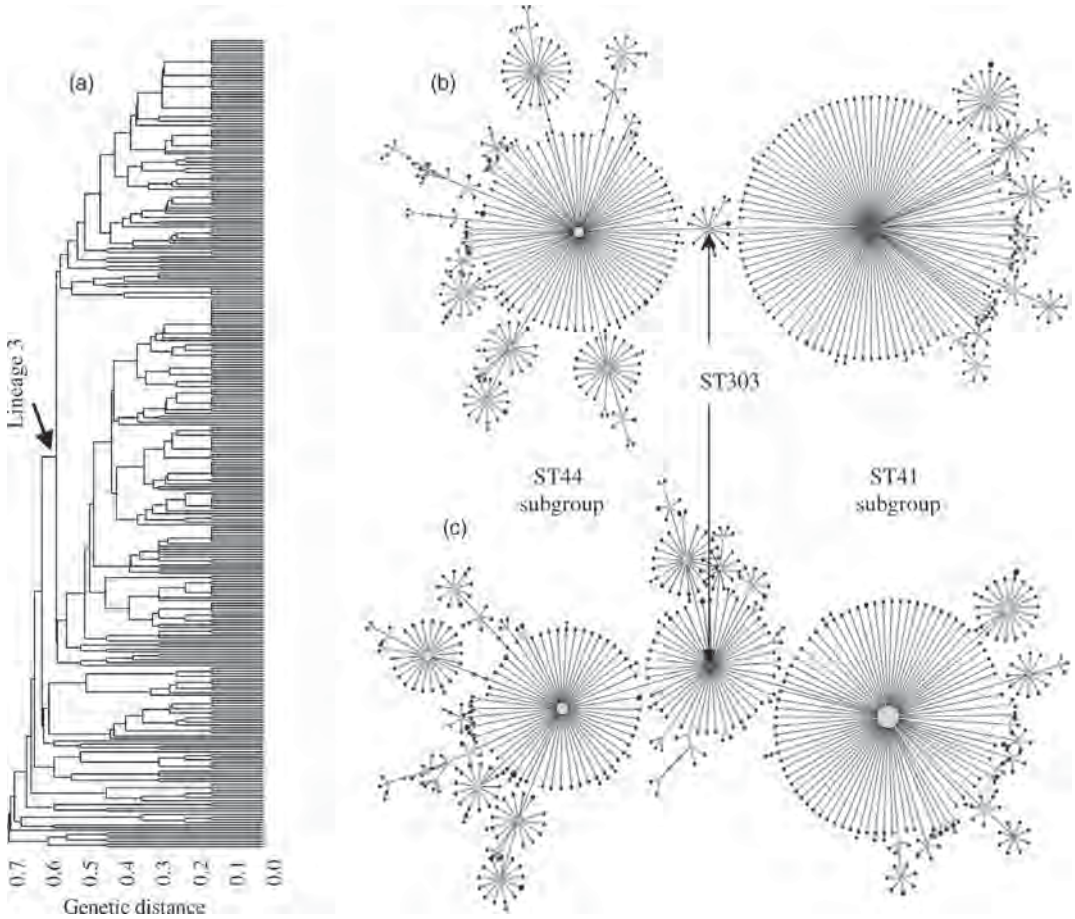

Fig. 29.3. Relatedness among isolates of lineage 3 of *N. meningitidis*. (a) A dendrogram based on differences in allelic profiles was used to display the relatedness among *N. meningitidis* STs (one example of each), and the node on the dendrogram from which the lineage 3 STs descend is shown (arrow). (b) eBURST was applied to all isolates in the *N. meningitidis* public MLST database (default settings) and the eBURST group containing lineage 3 was displayed as an eBURST diagram. (c) The eBURST diagram was redrawn with ST303 as the user-defined founder of the clonal complex. The ability to redraw diagrams with a user-defined founder, and automated editing of eBURST diagrams, are new features implemented in the current version of eBURST at the MLST web site (http://eburst.mlst.net).

large clonal complex (Fig. 29.3b). The two linked clusters of STs identified on the tree appear as two large subgroups, each with a predicted subgroup founder (ST41 and ST44), and a large set of SLVs (plus DLVs, etc). Interestingly, these two predicted subgroup founders are both SLVs of ST303, which is therefore phylogenetically central. One possibility is that ST303 is the founder of the whole clonal complex, but is now rarely encountered in contemporary samples of the meningococcal population, and has given rise to two successful SLVs, which have become founders of large subgroups within the clonal complex and have diversified extensively [15].

The hypothesis that ST303 may be the real founder of the clonal complex can be further explored. STs can be SLVs of more than one ST and, in building an eBURST diagram, the algorithm starts by predicting the founder of the clonal complex, as the ST with the greatest number of SLVs (ST41, in this case), and all of these SLVs are linked to ST41. Those STs that have not already been assigned to ST41 are then used to identify the ST with the second largest number of SLVs (ST44), and so on. This preferential assignment procedure results in a substantial underestimate in the eBURST diagram of the true number of SLVs of ST303, as many SLVs of this ST are also SLVs of ST41 or ST44, and have been preferentially assigned to these major subgroup founders. The eBURST diagram can be redrawn so that ST303 is the user-assigned ancestor of the clonal complex and all of its SLVs are then preferentially assigned to it (Fig. 29.3c). The resulting eBURST diagram shows that although ST303 is rare in the MLST database (there are only three isolates), it does have a large number of SLVs, and is the founder of a much more prominent subgroup than suggested by the initial eBURST diagram, which is consistent with it being the near extinct founder of this large clonal complex.

The stringent definition of an eBURST group can be changed from the default setting. Relaxing the definition, so

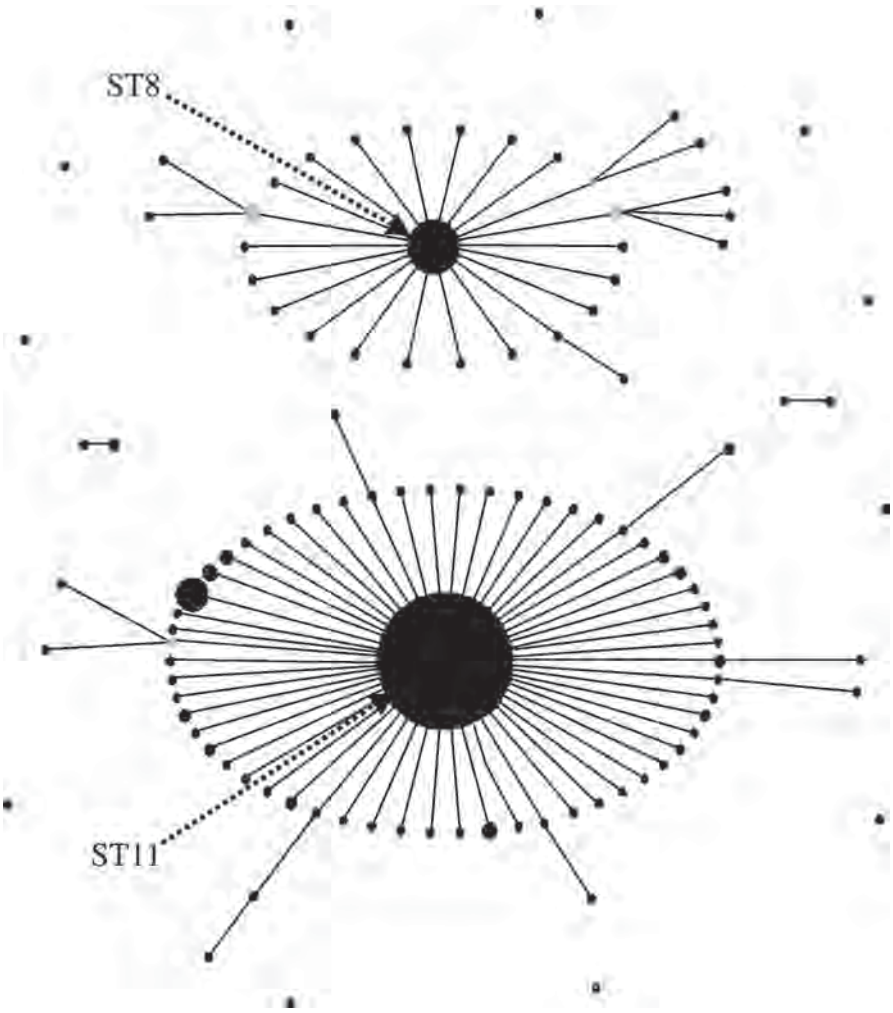

Fig. 29.4. Relatedness of *N. meningitidis* clonal complexes. Isolates assigned to the ST8 and ST11 clonal complexes of *N. meningitidis* are related and are grouped together using the less stringent group definition (sharing of 5/7 alleles) (Reproduced with permission from [15]).

that each ST in a group must share five or more alleles with at least one other member of the group, produces groups where some STs may have a low level of similarity in their allelic profiles, with much less confidence that they are all descended from a single founding ST that is still present in the population. The effect of relaxing the group definition is shown in Figure 29.4. In this example, two major unlinked clonal complexes of *N. meningitidis* (the ST8 and ST11 complexes) are placed within a single eBURST group within which there are also several unlinked individual STs. With this relaxed group definition, the unlinked STs must be DLVs of at least one ST in the group, but they cannot be SLVs of any ST, as otherwise they would have been linked. Similarly, no ST in the ST8 clonal complex can be a SLV of any ST in the ST11 complex; otherwise they would be linked through this SLV to form a single clonal complex. With the stringent default group definition the ST8 and ST11 clonal complexes evident in Figure 29.4 are in separate eBURST groups and their predicted founders are both assigned with 100% bootstrap support. The relaxed group definition highlights the fact that the ST8 and ST11 clonal complexes are likely to be related, and may well have descended from the same common ancestor, although no attempt is made to explore the nature of this possible common ancestor of the group [15].

As a ST can be a SLV of more than one ST, there are often several alternative eBURST diagrams that differ slightly in the arrangement of STs, and additional data (e.g., serotypes, drug resistance, etc.) can be mapped onto the eBURST diagram to decide whether an alternative pattern of local descent is more plausible. A complicated eBURST diagram is therefore a consensus view of a plausible hypothesis about the patterns of descent of the STs, in the same way that a phylogenetic tree is typically only one of many very similar trees that differ slightly in the branching order of the taxa. The current version of the program can highlight on the eBURST diagram all of the SLVs and DLVs of any selected ST, allowing possible alternative patterns of descent to be explored.

As MLST datasets become very large, clonal complexes may join into larger groups due to the chance presence of a ST that links previously unlinked clonal complexes. Distinguishing whether this congealing process reflects a real common ancestry between the clonal complexes, or is spurious, is problematic. Thus, the ST8 and ST11 meningococcal clonal complexes are considered to be related, although at the time their ancestry and patterns of descent were explored using eBURST [15], they were in separate eBURST groups using the stringent default group definition. Recently, the introduction of a new ST into the MLST database has resulted in the linking of these two clonal complexes – a SLV of ST11 is now linked through this new ST to a SLV of ST8. The new ST is phylogenetically central and could be considered as an ancestor for the whole group, although the fact that it is a unique recent isolate, and has very few SLVs, makes it unlikely.

29.2.2.2 Minimum spanning trees and split decomposition The minimum spanning tree (MST) method, implemented within the Bionumerics suite of programs (Applied Math, Sint-Martens-Latem, Belgium), is another new approach that can be used to explore the relatedness between groups of similar STs. The method uses the matrix of all pairwise distances between STs (based on the number of differences in their allelic profiles) and constructs a tree that minimizes the total distance between all STs. The method is most appropriate for recent pathways of descent as, by attempting to link all isolates in the input dataset, it assumes that ancestral and derived genotypes are still present in the sample. This is a realistic assumption for young clonal complexes (and is also an implicit assumption for defining the founder ST of a clonal complex using eBURST), but is an unrealistic expectation if highly divergent genotypes are analyzed, as the ancient common ancestors of divergent STs will no longer be represented in contemporary samples of the population. Even with isolates that have similar allelic profiles, implying descent from a recent common ancestor, there will be intermediate genotypes that may exist in the population but were unsampled, and the MST method allows these hypothetical intermediate genotypes to be postulated, and used to link all of the STs into a single tree. The MST method typically provides a number of alternative tree topologies that have the same minimum total distance and "priority rules" are used to select the most plausible tree. These rules are derived from the simple evolutionary model of clonal diversification used in the eBURST approach. Thus, the ST selected as the central (root) node of the MST, or the STs selected as secondary nodes, are identified as those that have the greatest numbers of SLVs. The tree produced by MST from a set of closely related STs should therefore be similar to the output of eBURST, with a founding ST (or root node), and founders of subgroups, with radial links to their SLVs, but it will be much less conservative and will link together these groups of STs using hypothetical intermediate STs. In contrast, eBURST does not even link a DLV of a founder ST to a clonal complex if the intermediate SLV on the path from the founder to the DLV is absent from the input data.

Split decomposition can be applied to allelic profiles as well as concatenated sequences and can explore alternative pathways between closely related STs. The algorithm will identify STs that have a large number of SLVs and the splits graph will show the radiation of these SLVs from their likely founder ST.

29.2.3 Comparing Split Decomposition, Minimum Spanning Trees and eBURST

For simple clonal complexes, the outputs from the MST method and eBURST are essentially identical, as the priority rules in the former incorporate the eBURST approach, and identify the ST with the greatest number of SLVs as the root node in the tree (Fig. 29.5a). With simple data the splits graph is also similar to that produced by eBURST and MST, and all three methods are far more informative than a tree (Fig. 29.5b,c). One difference is that a DLV of a predicted founder ST is linked to the founder on a splits graph, even when the linking SLV is absent from the dataset (the long branch indicating the ST is a DLV rather than a SLV of the founder), but is not linked in eBURST. Also, where two SLVs are equally likely to have given rise to a DLV, this is shown in the splits graph, as the DLV is joined to both of these SLVs (Fig. 29.5a). In eBURST this ambiguity is not shown, although the information is available by highlighting the DLV in the eBURST diagram and selecting the option to show all of its SLVs. For larger clonal complexes, and clonal complexes with several subgroups, the MST method and split decomposition are much less informative than eBURST (Fig. 29.6c). For example, the splits graph produced from lineage 3 (Fig. 29.6a) gives a poor representation of the possible pathways of descent in this large clonal complex with no indication of any subgroup structure. The MST is better as by incorporating the eBURST approach within the algorithm it does detect subgroups (Fig. 29.6b).

The eBURST, Splits Tree, and MST methods can be applied to other types of digital data, including MLEE data. For example, unambiguous characterization of isolates of pathogens that genetically are too uniform to apply MLST is increasingly performed using variable number tandem repeats (VNTRs) [21,26]. In this approach the number or repeats at a locus in each isolate is estimated accurately using a capillary DNA sequencer, and the number of repeats present at 10–20 loci provides a string of integers, which defines each isolate. eBURST can identify groups of isolates with a user-defined number of shared repeat lengths and can predict the founding pattern of repeat length polymorphisms. The predicted patterns of descent from the founder to the other isolates could then be displayed, although modifications of the existing software are needed as linking of isolates that differ at more that one locus may be appropriate where large numbers of VNTRs are used. The MST and split decomposition methods are directly applicable, as the VNTR profiles can be connected, even if linking profiles are absent from the dataset.

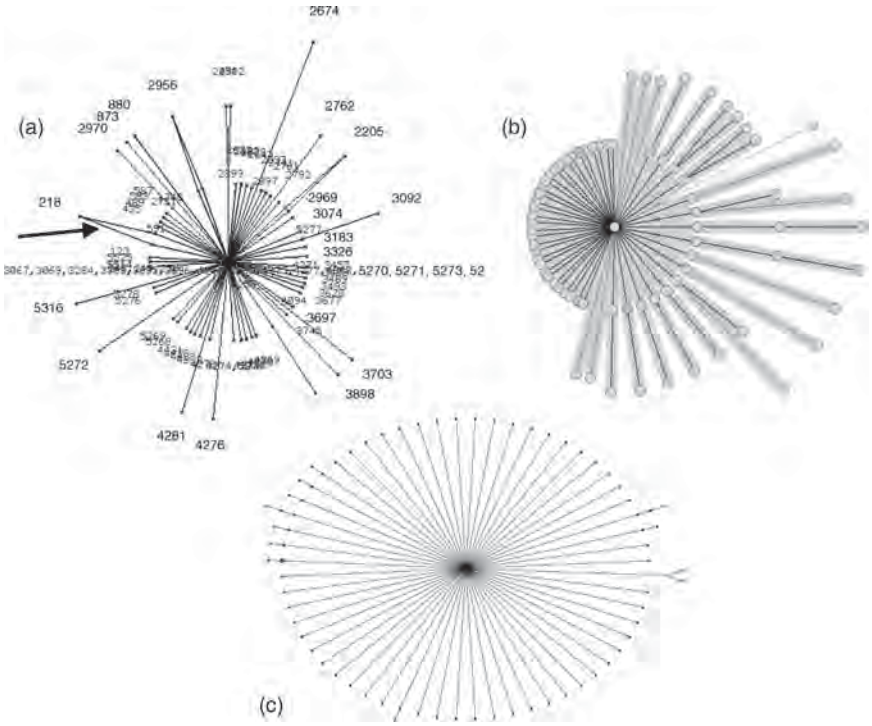

Fig. 29.5. Different representations of the relatedness of STs. STs that shared four or more alleles with ST213 of *N. meningitidis* were extracted from the public MLST database and the relationships among these were displayed using split decomposition (a), MST (b), and eBURST (c). The great majority of STs are SLVs of ST213 and all three methods provide an adequate representation of this simple clonal complex. The arrow in (a) shows the ability of split decomposition to show alternative routes to a DLV. Some STs are not shown in the eBURST diagram as this method is conservative and only links STs that differ at a single locus.

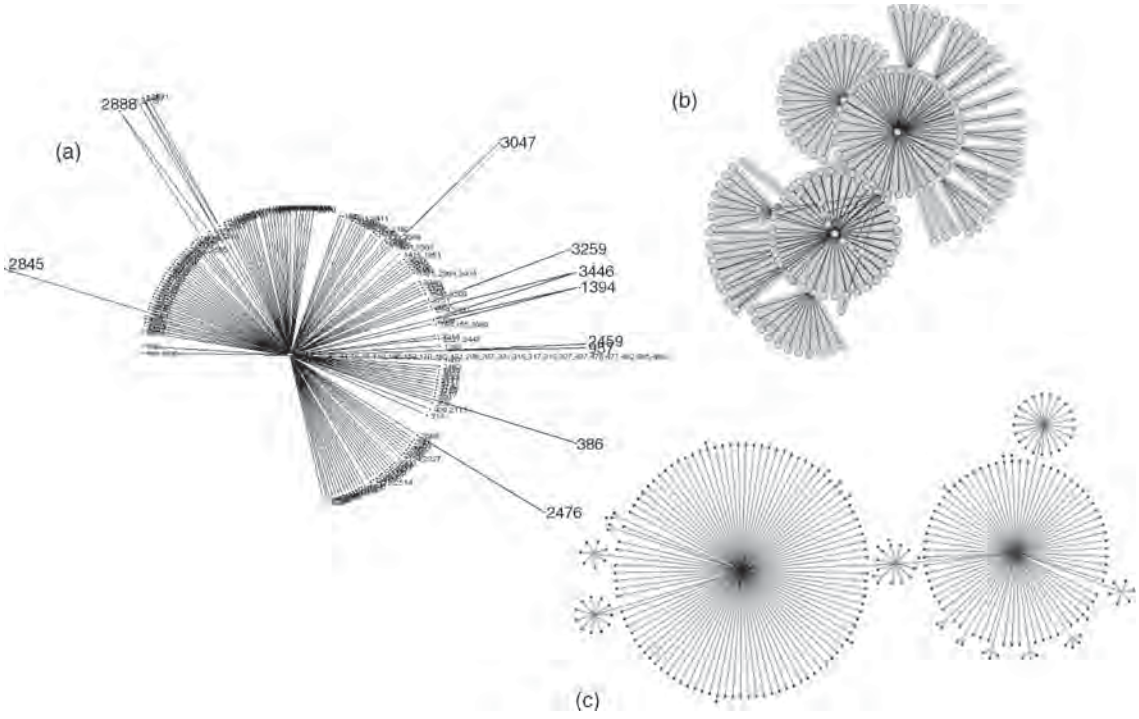

Fig. 29.6. Representation of *N. meningitidis* lineage 3 using three methods. STs of lineage 3 displayed by split decomposition (a), MST (b), and eBURST (c). To reduce the number of STs, only those that share four of more alleles with ST303 were extracted from the MLST database; the eBURST diagram is therefore less complex than that shown in Figure 29.3.

These procedures for exploring recent ancestry and patterns of descent are relatively new, and their reliability and limitations need to be explored using realistic simulated populations, where the real patterns of descent are known (see Section 29.3). However, they almost certainly will produce a much more informative and plausible view of the relatedness among similar isolates than a standard dendrogram that uses the differences in the allelic profiles of the isolates. However, eBURST diagrams, MSTs, or splits graphs should not be overinterpreted and all need to be treated as hypotheses about descent that can be further explored.

29.2.4 Displaying the Overall Structure of a Population

eBURST allows all of the STs within a bacterial population to be displayed in a single diagram. This "population snapshot" provides a useful overview of an entire population, displaying all of the clonal complexes, and the unlinked STs, although comparing the structures of different populations or species in this way requires that careful attention is paid to sampling issues [15]. Figure 29.7 shows a population snapshot of the isolates within one of the smaller MLST databases (*Haemophilus influenzae*), but population snapshots can also be applied to databases which include many thousands of isolates. The MST method can also show the relatedness of all STs, and can link them into a single diagram, using hypothetical intermediates, but it is very unclear that the relationships between linked clusters are meaningful, except perhaps in highly clonal species.

29.3 MLST DATA AS A RESOURCE FOR BACTERIAL POPULATION GENETICS

A current challenge in bacterial population genetics is to find new methods and tools that allow the large amounts of data being produced by MLST (and other similar methods) to provide new insights into the mode and tempo of bacterial evolution. Some progress has been made, particularly in addressing the extent and impact of recombination in different bacterial pathogens, but new approaches designed to capitalize on the type of data produced by MLST are required, over and above the standard population genetic statistics that can be applied to MLST data.

An important aim of population genetics is to understand the role of mutation, recombination, genetic drift, selection, and so on in generating the observed patterns of genetic variation in populations. In principle, the distribution of STs within a well-sampled population should provide information about the processes that have generated this distribution, and a substantial body of population genetic theory for both sexual and asexual organisms is available for this purpose. The problem

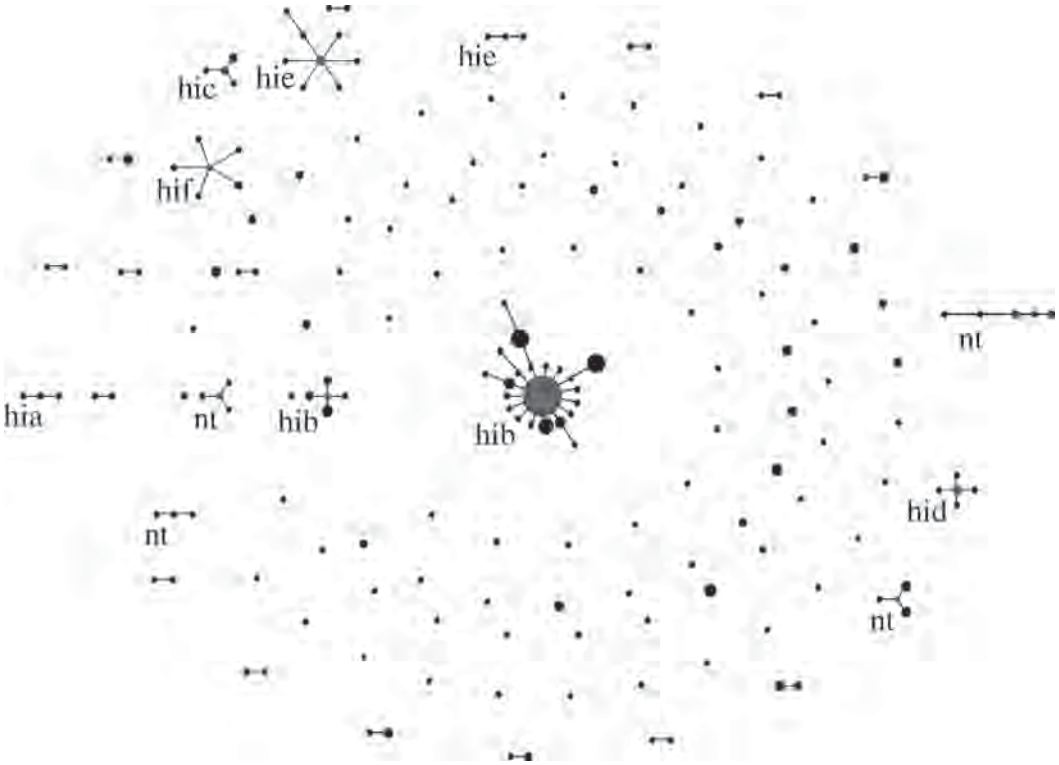

Fig. 29.7. Population snapshot of the *H. influenzae* MLST database. All isolates in the *H. influenzae* MLST database were displayed as an eBURST group, which shows all of the clonal complexes, and the unlinked STs in the population. The major clonal complex containing isolates of the different capsular serotypes are shown (hia, serotype a; hib, serotype b, and so on; nt, nontypeable).

with bacteria is that they are not sexual (in the eukaryotic sense, where alleles in the population are redistributed in each generation by meiosis), or truly asexual, and they fall within a genetic hinterland where neither the theory for sexual nor asexual organisms can be applied. To make matters worse, the extent of recombination in different bacterial species varies greatly and in most cases we have little knowledge of the extent of recombination in the species we wish to study. Population genetic theory, therefore, needs to be developed that can accommodate these special and variable features of bacterial sex [12], and also for eukaryotic pathogens and parasites that have unusual sex lives [35]. For bacteria, population genetic models should be able to take advantage of the type of data being produced by MLST and recently there have been developments in producing multilocus models for bacterial populations that incorporate variable levels of recombination.

Models need to be tested by comparing their output to real data and a key issue in the analysis of bacterial populations is appropriate sampling, so that model outputs are fitted to real populations that accurately represent those that are transmitted to new hosts. Many studies that analyze populations of bacterial pathogens have focused on isolates from disease. This may be appropriate for pathogens where asymptomatic infection is rare and isolates from disease are representative of the population, but there are many bacterial pathogens where infection is common but disease is rare. Some of the best examples are provided by "accidental pathogens," such as *N. meningitidis*, *Streptococcus pneumoniae*, or *Staphylococcus aureus*, where colonization is frequent but rarely leads to disease. Thus, approximately 10% of children and young adults in Europe or North America carry *N. meningitidis* in their nasopharynx, even though only a handful of episodes of meningococcal disease typically occur per 100,000 of population. Isolates from disease are therefore totally unrepresentative of the natural populations of these pathogens and an unbiased sample of the population requires isolates from asymptomatic carriage. In fact, disease may be largely irrelevant to the long-term evolution of the pathogen as transmission to new hosts occurs from the nasopharynx, and the invasion of the blood and/or cerebrospinal fluid, leading to meningococcal disease in a small minority of infected hosts, is very unlikely to result in increased transmission of these bacteria to new hosts.

An analytical multilocus model that incorporates recombination has recently been developed by Fraser et al. [16]. This is basically a neutral Wright-Fisher model with nonoverlapping generations in which the set of multilocus genotypes present at time t are sampled to produce population $t + 1$, with changes in alleles occurring between generations by mutation (generating new alleles) or by recombination (replacement of an allele with another allele from within the population). The model output matches the distribution of STs in real populations surprising well, but it significantly underestimates the proportion of pairs of identical isolates that are observed in real populations from a single geographic region. This excess of identical isolates is best explained by epidemiological processes that in directly transmitted pathogens lead to the local spread of certain genotypes [16].

This clustering of genotypes in local populations probably occurs as human contact patterns are highly heterogeneous, with transmission of a strain being much more likely within families, or to colleagues at work, than to casual contacts or the wider community. These transmission processes will leave their imprint on the distribution of genotypes within populations, although methods are required to quantify these processes, and to distinguish clustering due to heterogeneities in transmission from those resulting from fitness differences (i.e., selection). The observed patterns of clustering within a well-sampled local population should provide information about the process of transmission. Thus, where transmission to new hosts is very rare (e.g., *H. pylori*), there are expected to be few clusters of indistinguishable genotypes in a local sample of the pathogen population, whereas in those species in which transmission is frequent (e.g., *N. meningitidis*) there should be much more clustering. The analytical model of Fraser et al., therefore, incorporates a clustering parameter, which greatly improves the fit of the model to data, and provides estimates of the population mutation and recombination rate, and the extent of clustering, from the observed distribution of STs in a well-sampled population of a directly transmitted bacterial pathogen.

A major issue that needs to be addressed is whether the distribution of STs in a well-sampled bacterial population is largely the result of neutral (i.e., stochastic) processes, or if there are substantial fitness differences among strains of a pathogen, so that genotypes increase and decrease in prevalence due to natural selection. Distinguishing between these possibilities is not trivial, as current tests for neutrality lack sensitivity, and the imprint on the population of selection may not easily be distinguished from other processes. The basic model of Fraser et al. [16] provides a null hypothesis for the expected distribution of multilocus genotypes within a bacterial population under neutrality. The observed distribution of genotypes in a real population can be compared to that expected under the null hypothesis of neutrality and, if it deviates significantly from neutrality, the reasons for this can be explored in terms of epidemiological clustering, selection, and so on [18].

One advantage of having a model of bacterial evolution is that simulated bacterial populations can be produced for any species where there are adequate real data to estimate the parameters of the model (mutation rate, recombination rate, and extent of epidemiological clustering). Realistic simulation will allow different approaches to identifying clonal complexes and patterns of descent to be critically evaluated.

29.4 MEASURING RATES OF RECOMBINATION FROM MLST DATA

There has been considerable interest in exploring the extent of recombination in bacterial pathogens and a number of methods (e.g., measures of linkage disequilibrium) have been

applied to data from MLEE and MLST [12]. Most of these approaches have been semiquantitative, at best, and have indicated that some bacteria appear to be highly recombinogenic whereas others are much less so. More recently, there have been attempts to measure rates of recombination in different species, or the relative contributions of recombination compared to point mutation in introducing genetic variation at a locus. The sequences obtained using MLST, and the distribution of multilocus genotypes within a well-sampled population, provide data from which, in principle, it is possible to estimate rates of recombination. The most direct approach is to analyze the allelic changes that occur as the founder ST of a clonal complex diversifies to form SLVs [11]. The sequence changes between the ancestral allele in the founder ST, and the variant allele in each SLV, are scored as being the result of either a point mutation, or a recombinational exchange. Summing the results from all of the SLVs provides a ratio of the number of changes at alleles (or individual nucleotide sites) that occur by recombination, compared to the number that occur by point mutation, as clones start to diversify [11]. This approach has indicated that evolutionary change at housekeeping loci may occur in some species at least ten times more commonly than point mutation, whereas in others mutation appears to be at least ten times more common than recombination [14]. The attraction of this method is that it scores individual incidences where recent changes at a locus have occurred, rather than extracting information indirectly from sets of sequences or multilocus genotypes, although simplifying assumptions have to be made when assigning allelic change to recombination or point mutation [11].

Coalescent approaches can also be applied to estimate the rate of recombination from the concatenated sequences of the MLST loci [23,33]. The analytical model of Fraser et al. [16] also estimates the recombination to mutation rate from MLST data, but uses a completely different approach to that of Feil et al. [11], and provides an independent estimate of this parameter. There is a need to evaluate the reliability of methods which estimate rates of recombination from sequence data, or from distributions of multilocus genotypes, and to see how they correlate with rates of recombination obtained from the more direct approach of scoring allelic changes as recombinational or mutational events.

29.5 CONCLUDING REMARKS

MLST has been widely used since its introduction in 1998 and has provided a powerful new tool for epidemiological studies. The clear evidence for a major impact of recombination in many bacterial species makes it unwise to use concatenated sequences of MLST loci to infer deeper relationships between lineages, unless the impact of recombination in the species has been shown to be minor, and has focused attention on better methods of identifying clusters of related isolates, their founding genotypes, and patterns of recent evolutionary descent. The increasing availability of MLST data is stimulating new approaches to addressing aspects of the population biology of bacteria. This trend is likely to continue and hopefully will lead to a much better understanding of the evolution of bacterial populations and of fungal pathogens. The method is also being introduced by microbial ecologists and promises to provide a better understanding of the population biology of environmental species and to inform the debate about the nature and extent of geographic substructuring in such microbes.

GLOSSARY

Dendrogram: A branching diagram, resembling a tree, that shows the clustering of strains based on their similarities and differences. The distance along the tree from one strain to the next represents their relative degree of similarity.

Phylogenetic tree: This differs from a dendrogram as, although it also shows the similarities among strains, the branching patterns represent the inferred evolutionary relatedness between strains derived from a particular model of the evolutionary process.

Genetic distance: A quantitative measure of the amount of genetic difference between strains, typically on a scale of 0 (identical) to 1 (100% different).

Coalescent approaches: The coalescent considers a sample of alleles (sequences) and infers information about the processes (mutation, recombination, etc.) that generated the sample by working backwards in time to the common ancestor. The term "coalescence" refers to the point at which, as we move backward in time, the genealogy of alleles merges or "coalesces," at the point where they share a common ancestor.

Wright-Fisher model: A model of random genetic drift, in which the genotypes of the next generation are determined by randomly sampling alleles from the present one.

Clone: Strictly speaking, a clone is a lineage of genetically identical individuals that arises by asexual reproduction. In bacteriology, it is generally defined more loosely as a set of isolates that are indistinguishable by the molecular typing method being used, due to their recent descent from the same common ancestor. For example, using MLST, isolates with the same allelic profile are assigned as members of the same clone and the term is essentially synonymous with "strain" or "sequence type."

Clonal: Bacteria divide to produce two essentially identical descendants. This process of binary fission leads to independently evolving asexual (or clonal) lineages in which variation gradually accumulates by the process of mutation.

Sexual: Bacteria do not have sexual reproduction as typified by higher organisms, but they are not completely asexual, because there are genetic mechanisms that result in the replacement of

small regions of a bacterial chromosome with the corresponding regions from other isolates of the species (recombination). These recombinational replacements may be rare, or they may be frequent, leading to difference among bacterial species in the relative contribution of mutation and recombination to the genetic diversification of strains. If recombination is rare the population is said to be highly clonal, whereas if recombination is very common it is considered to be non-clonal, or sexual.

Recombination: An exchange of DNA. In this chapter, recombination refers to the process by which small segments of the bacterial chromosome are replaced by the corresponding segment from another strain of the species (or from a very closely related species). This is homologous recombination as the segment that is replaced and the replacing segment are very similar in nucleotide sequence.

Variable number tandem repeats (VNTRs): Bacterial chromosomal DNA typically contains a number of simple tandem repeat sequences, such as (ATCCGG)*n*, and errors during replication of the DNA can increase or decrease the number of copies (*n*) of the repeat. The number of copies, therefore, varies among strains of a species, and by measuring *n* for 10–20 different tandemly repeated sequences a strain can be precisely characterized as a string of integers.

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CHAPTER 30

TOPICAL DEBATES

Evaluation of Risks and Benefits of Consumption of Antibiotics: From Individual to Public Health

Fernando Baquero

Department of Microbiology, Ramón y Cajal University Hospital, C. Colmenar km 9,100. 28034 Madrid, Spain

30.1 ANTIBIOTICS AND HUMAN HEALTH

Antibiotics can be considered one of the more influential human discoveries affecting human health. Two aspects are critical to understand such impact. First, antibiotics have contributed to a significant decrease in the individual morbidity and mortality of classic infectious diseases, even though many of these diseases started to decrease in incidence in advanced nations before the introduction of antibiotics, probably because of improved sanitation and social changes. Second, antibiotics have served as catalizers in the development of modern Medicine, assuring a protective umbrella against infections in advanced surgery procedures, immunodefficient conditions (including therapy), or in intensive-care facilities. Indeed, the preservation of the activity of antibiotics remains at the present time a condition for the progress of Medicine. Interestingly, as it was pointed out by Stuart Levy in his book "The Antibiotic Paradox" [25], because of the selection of naturally emerging antibiotic-resistant bacteria, the extended use of antibiotics erodes its own action, and facing this possibility, only two issues remain possible: to decrease any excess in consumption of these drugs, or to maintain very active research against infectious diseases, either stimulating the discovery of new antibiotics or searching for alternative strategies.

30.2 THE DETERMINANTS OF HEALTH: CONSERVATION MEDICINE

Medicine should be more and more concerned with the assessment of the determinants of health [28]. Changes in disease prevalence are associated with human behavior. Conservation medicine can be defined as the study of the consequences of the human footprint on the ecological balance of disease dynamics, and the ways of controlling such anthropogenic impact [1,22]. Conservation medicine is a synthetic discipline operating at biological scales ranging from the molecular to ecological health issues, but might be also considered as a crisis discipline [22], emerging in a moment of human history in which the increasing technological capability of our species is suspected to start to be pathogenic. Simultaneously, fascination with health is greater than ever, and research of hazards, conveyed to the public by the media and the institutions, has become a major psychological component of the individuals of a society claiming for personal behavior and self-realization [17]. We assume that the consumption of antimicrobial agents is certainly a factor deeply influencing public health, but that factor is mainly originated in human behavior [25]. Rooted in behavioral ecology, conservation medicine may reach some quantitative understanding of health (Consortium for Conservation Medicine, http://www.conservationmedicine.org).

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

30.3 FROM FEARS TO POSSIBILITIES

Nobody discusses about the large benefits for health derived from the use of antibiotics, even though these benefits have never been properly quantified. Nevertheless, only knowing the benefits, the risks associated with a loss of its potency can be calculated. Conversely, everybody discuss about the risks of antibiotic use. In general, the predominant fears about the use of antibiotics are expressed in the following ways: (i) they can select more and more antibiotic-resistant organisms, and therefore a point will arrive in which infections could not be cured anymore; and (ii) there are important toxic effects eventually associated to antibiotic use. Microbiologists have provided a key element to quantify the more easy-to-measure undesirable effect of antibiotics, that is, antibiotic resistance detected in susceptibility testing. For a number of bacterial organisms, a solid correlation between antibiotic consumption and antibiotic resistance has also been documented. Consequently, antibiotic policies have been implemented to decrease antibiotic use in order to reduce the intensity of selection for antibiotic-resistant bacteria. This is certainly a simple, easy-to-measure, objective and reasonable task. Unfortunately, the consequences for human health both of this action (stringent control of antibiotic use), or the lack of action (relaxed control of antibiotic use), remain largely unknown. The epistemological problem becomes evident when the public health authorities naively propose the question: What is the proven consequences of antibiotic resistance for public health? Note that the question cannot be answered just proudly showing impressive curves of relations between antibiotic use and resistance. At the present time we should honestly answer: "We do not know, but there is a reasonable guess." The problem is if some interventions can be only based on reasonable guesses, frequently taken by managers, economists, and politicians as the real truth, and potentially leading to suboptimal allocation of resources [33]. As it has recently stated for complex situations, "Zealots who preach a particular answer with unfounded but unshakeable belief often make these problems worse" [26]. Probably many significant discoveries in antibiotics would become limited or prevented by abuses in precautionary principles (http://www.spiked-online.com/sections/risk/index.htm). Without any doubt, the estimation of health consequences of the use of antibiotics is a complex problem, sufficiently complicated that only probabilistic statements can be made. Anyhow, as in other complex realities, scientific efforts should be urgently applied to obtain these probabilities, to be used by individuals, both lay and professional [21].

30.4 HOW IMPORTANT IS ANTIBIOTIC RESISTANCE AS A RISK FOR PUBLIC HEALTH?

When we are approaching a century of chemotherapy, after hundreds of millions of antibiotic treatments, despite of many tons of wise scientific papers on antimicrobial resistance, we simply remain unable to answer this question in a precise scientific way. Of course we know that resistance can be important for the prognostic of particular cases, and therefore we guess that antibiotic resistance should be important for public health. But this perception of risk may be different for many of us. Microbiologists know that antibiotic resistant bacteria are selected by antibiotic use, and therefore the infections that they may produce should be less successfully treated than those caused by sensitive organisms, and therefore resistance should be a problem of public health. Pharmacologists take a more relativistic position: resistance should be only a problem if the pharmacokinetics/dynamics of the drug is not able to overcome resistance. Clinicians have different views about the risks associated to antibiotic resistance depending on their personal experiences with patients. General practitioners working in the community rarely feel the risk of resistance. Despite the fact that susceptible or resistant bacteria are isolated, most antibiotic-treated patients recover in the same or in very similar way. The reason is that most of the infections they attend are self-limited, and therefore a favorable outcome could be expected in most cases, even though the success is most frequently attributed to the antibiotic prescribed. This experience is also common in hospital-based practitioners; for instance, a common mixed strategy of antibiotics plus excision and appropriate drainage of the infected tissue cure most common surgical infections, caused either by susceptible or resistant organisms. In this case, excision and drainage by itself could have been equally effective. In severe infections occurring in patients with foreign bodies, or in critically ill patients, the clinical evolution may frequently appear to be independent from antibiotic susceptibility of the recovered organisms. In some cases, antibiotics might be quite ineffective in critical patients with overwhelming opportunistic invasions by susceptible organisms; in other cases, the antibiotic is unable to reach the infected (for instance avascular) site of infection. Eventually, the organisms recovered in cultures, either susceptible or resistant, have no causal relation with the real infection. Indeed, clinicians are frequently disappointed when they receive the report of a susceptible organism in a patient where antibiotic therapy failed, or of a resistant organism in a patient where therapy apparently succeeded. The perception of the risk is better in the rare cases where the same strain evolves during therapy from susceptibility to resistance, and that correlates with the worsening of symptoms. Interestingly, the perception of the risk of resistance increases with the accumulation of difficult-to-treat cases during epidemics of some infections caused resistant organisms, if the practitioner remember what was the expected outcome with the susceptible ones. These examples serve to illustrate the difficulties of the doctors in real life to get from their own experience an accurate feeling of the risks associated with antibiotic resistance.

30.5 HEALTH VERSUS RESISTANCE

Resistance is much more easily measured than health, and science, as any other flowing process tends to find its way through locus minoris resistentiae. In some cases, that leads to a critical deviation with consequences in the identification of the scientific target. It is not unusual to define the hazard associated with the abuse of antibiotics with the emergence and

spread of resistant organisms, or even of the resistance genetic determinants, instead of the real hazard, that is obviously a negative effect on human health. Indeed, resistance is only important because of its consequences in human health that we largely ignore. But human health is a very wide concept, and specification of the target of risk or benefit target is a desirable condition of any study [20]. We believe that the target that should be considered is the individual health risk or benefit of antibiotic consumers.

30.6 CHANGES IN ANTIBIOTIC CONSUMER'S BEHAVIOR: EGOISM VERSUS ALTRUISM

Any intervention trying to regulate the consumption of antibiotics to its appropriate level should be mainly based on changes in the behavior of antibiotic consumers: prescribers and patients. Of course public health authorities may in critical situations take stringent control measures, obliging to follow certain criteria, but in most modern societies this intervention will be only acceptable in quite exceptional cases. If the targets for changes in behavior are both prescribers and patients, they should be fully convinced of the value of taking personal attitudes toward antibiotic consumption. And this conviction is necessarily rooted in egoistic or altruistic feelings, or in a variable interaction between both attitudes [6,16].

30.7 THE ROLE OF WORRY IN INDIVIDUAL PATIENT'S BEHAVIOR

The individual is expected to adopt personal attitudes toward him and his/her family about the use of antibiotics based on the interest in shaping the best own personal future, and much less so because of the collective risk for other people [9]. We, scientists, should provide data to back the desirable change in attitudes, as worrying without data is the most characteristic prescientific manipulation of mankind. For such a purpose of measuring individual drug-induced effects, we are obliged to objectively evaluate the balance of risks and benefits [24], for each type of individual in the population associated to the consumption of antimicrobial agents during life. This knowledge is absolutely needed to create a scientifically based culture of use of antimicrobials, and educate and convince the patients, the prescribers, the companies, and the administration about eventual interventions. At long term, the building up of such a culture should have a positive impact on the individual consumer's psychology [34], and certainly will improve public health as a whole.

30.8 THE ROLE OF WORRY IN THE PRESCRIBER'S BEHAVIOR

In the case of the antibiotic prescriber (most frequently a general practitioner working in community medicine), the attitude toward an external recommendation of using antibiotics in a much more selective way may be a complex one. On one hand, the prescriber may accept that such a practice will be for the benefit of the patient's in general (public health), for instance, by reducing the resistance rates. But he may consider that restricting the use of antimicrobials to the very clear cases in which prescription is fully justified by objectively documented reasons might also increase a number of risks for his individual patient and for himself. A number of patients not fulfilling objective criteria for therapy may still have benefits from antibiotics. Severe, even deadly infections in preliminary stage (as bacterial meningitis or sepsis) could be aborted; subclinical infections (as urinary tract infections in young girls) may be cured; self-limited infections (as upper respiratory tract infections or otitis media) may be shortened in time or relieved in symptomatology. Moreover, if something wrong unexpectedly happens and the patient is not treated, the prescriber could be confronted with malpractice lawsuits.

30.9 INDIVIDUAL VERSUS SOCIETY COMPONENTS IN SHAPING INDIVIDUAL RISKS

We know that individual tobacco smoking is the main component increasing the individual risk for coronary artery disease or lung cancer, and we also accept the existence of a minor component of social behavior in passive smokers. Conversely, in the case of the risk associated with antibiotic individual hyper-consumption, it might be suggested that the main component could be the behavior of others. That seems to be particularly clear in the case of the emergence and acquisition of antibiotic-resistant organisms. The emergence and fixation of resistant variants (mutants) during a single short course of antibiotic therapy is probably low, either for the infecting or the commensal organisms. The conventional wisdom suggests that resistant organisms are mainly acquired "from others" and therefore the individual behavior in antibiotic consumption may play a minor role. This is not entirely true. The individual uptake of antibiotics increases the possibility of acquisition of resistant organisms from exogenous sources. On the contrary, if a single course of therapy has low influence in the emergence of resistant variants in the endogenous flora, repeated courses of therapy may efficiently select such variants [7]. Finally, it is essential to understand the real risk for the individuals is not the emergence or acquisition of antibiotic-resistant bacteria, but the effects that may result at short or long term of hosting such type of organisms.

The individual responsibility in shaping individual risks can be easily understood in those adverse effects resulting from the non-antibiotic action of the drug (as acute or chronic toxicity, effects derived from drug–drug interactions, or hypersensibility). The same is true for the antibiotic effects resulting in an alteration of the physiological role of the normal microbiota associate to the individual.

30.10 APPROPRIATE DEMAND OF ANTIBIOTICS AND THE INDIVIDUAL RISK

It is essential to demonstrate that antibiotics are absolutely needed to maintain high health quality in modern societies. In the cases in which antibiotics are indicated, the benefits overweight by far the risks, including antibiotic resistance. Because of that, we should propose that only the excess in use of antibiotics constitutes a dangerous practice for public health. The question is how to quantify and control such an excess. In collective terms, we should be able to construct, for each particular area and period of time, a theoretical "line of appropriate use of antibiotics," correlating (in abscissa) the number of infections in which antibiotics are indicated (there is a grounded expectation about a positive intervention in the outcome of the infection), and the rate of utilization of antibiotics. For instance, during an epidemics (increase of the number of infections) the use of antibiotics may also increase in a totally justified way. If that increase does not occur, may be there is an under-prescription of antibiotics. If an increase occurs without any associated increase in the number of cases, other factors unrelated with justified demand are influencing the prescription trends, and measures should be taken to control them.

The use of the parameter "number of infections in which antibiotics are indicated" is just an approximation, that should be further refined. For instance, the indication of therapy of a pneumococcal pneumonia by a macrolide may exist, but this indication should be modulated by the local prevalence of macrolide resistance in *S. pneumoniae* increasing the expected failure rate (the indication exists in general, but may be not in a particular location). The severity of infection (including chronic infections) should also be considered: patients with severe or chronic infections receive (in a justified way) more antibiotics than patients with acute, milder infections. It could be useful to built an integrated parameter considering these facts, to better correlate antibiotic consumption with real justified needs. This parameter should reflect the appropriate demand for antibiotics (ADA) in a particular time/space frame [5].

30.11 "MY" UTILIZATION OF ANTIBIOTICS: A PERSONAL DECISION

In the next future, we should be able to answer the following question that every individual has the right to formulate in his own or his family's responsibility: "How important is the excess risk for my personal health resulting from my personal level of consumption of antimicrobial agents?" The answer should be of the following type: "If you belongs to this particular (x) group of individuals, and your level of consumption is of a certain (y), your possibilities of current (r_1) and projected (r_2) excess risks for your personal health are of: $0 \leq (r_1 \text{ or } r_2) \leq 1$. The secondary question: "How important are the excess risk derived from the level of consumption in others?" should be answered in a similar way. The probabilities will be identified inductively for each (x,y) cell in the resulting multidimensional contingency table. In a symmetric way, the same questions should be considered by the prescriber: "How important is the excess risk for the health of this particular patient resulting from the prescription of antimicrobial agents that I am considering for this individual?" Note that the same type of questions and answers have entered in the routine medical practice in our days for tobacco smoking, cholesterol levels, or hypertension in coronary artery disease. The only apparent difference is that in the case of antimicrobials, the "excess risk" might be considered negative; for instance, the patient could have a benefit from a presumed excessive use of antimicrobials. The individuals, either patients or prescribers, are expected to arrive at their own personal balance between the risks and benefits [4].

30.12 THE INDIVIDUAL HEALTH RISKS OF ANTIBIOTIC USE

In a first group, we should consider those negative events presumptively related with an increase of antibiotic resistance due to the individual overexposure to antibiotics.

1. Increase in clinical failure in the therapy of current and future bacterial infections.
2. Suboptimal recovery from current and future bacterial infections.
3. Increase in metastatic bacterial infections.
4. Increase in recurrence rates and chronicity after bacterial acute infection.
5. Increase in transmission rate of bacterial organisms to the familiar neighborhood.
6. Increase in current or future opportunistic infections with resistant organisms.
7. Increase in current or future bacterial complications of trauma, surgery, and therapeutic or pathological immunodepression.
8. Increase in superinfections by resistant organisms.
9. Increase in para-bacterial diseases. An obvious example is the possibility of increase of infections like rheumatic fever or nephritis because of sub-optimal therapy of streptococcal infections.

A second group of negative events of individual increased antibiotic exposure includes those related with the antibiotic-derived effects on the host's physiology, including those derived from the alteration of the normal microbiota associated to human surfaces.

1. Increase in acute episodes of antibiotic toxicity, including hypersensibility.
2. Increase in undesirable effects associated with drug–drug interactions (for instance sudden death by associating macrolides and inhibitors of cytochrome 450 3A; [31]).

3. Increase in chronic toxic effects on human cell lines, including mutagenicity. For instance, the use of antibiotics reduces the ability of human microbiota to metabolize phytochemicals into compounds that may protect against cancer, or might alter the effect of intestinal microbes on human hormones influencing carcinogenicity. Indeed the use of antibiotics might be associated to the risk of breast cancer [37].

The evaluation of these possible negative events of increased antibiotic exposure should be counterbalanced with the possible benefits. Note that these positive events are not merely the mirror image of the absence of the negative ones.

30.13 THE INDIVIDUAL HEALTH BENEFITS OF ANTIBIOTIC USE

1. Increase in clinical cure or substantial recovery of a number of infections.
2. Decrease in time of disability because of infection, with increase in working productivity and/or quality of life.
3. Decrease in the number of severe acute infections eventually complicating apparently mild infections (such as meningococcal meningitis or pneumococcal pneumonia).
4. Decrease in subclinical infections with high health impact such as acne, caries, periodontitis, or subclinical urinary tract infections in young women.
5. Decrease in chronicity after acute infection, or in the number of recurrences in chronic infections.
6. Decrease in transmission of pathogenic or resistant bacteria to the close neighborhood (as *S. pyogenes*, or *C. trachomatis* [11]).
7. Decrease in the number of meta-infective diseases, as rheumatic fever.
8. Decrease in the number of para-microbial diseases eventually associated with acute or chronic mild diseases (cardiovascular diseases, rheumatic arthritis) [10].
9. Decrease in carcinogenicity, or in central nervous system diseases (as with tetracyclines) [15].

30.14 THE PROBLEM OF MINIMUMS: MINIMAL BENEFITS VERSUS MINIMAL RISKS

A typical problem in practical chemotherapy is the problem of minimums. Should an antibiotic be prescribed in a situation in which a minimal benefit is expected from such an use, but at the expenses of only a minimal risk? Such small benefits are only detectable using very large series of individuals, and therefore a parameter, the number needed to treat for a benefit (NNTB), is used. For instance, it has been claimed that antibiotics should not be used for acute otitis media, as NNTB is only 15 [18]. On the contrary, the use in acute purulent rhinitis has an NNBT of 6–8, and NNBT of 5 was found for acute bronchitis [3,35]. For streptococcal tonsillitis in developed countries, reduction of 16 h in symptoms (pain, that can be determined in infants by the "last day of crying") occurs for an NNTB of 2–7 [3]. Nevertheless, many experts do not recommend the use of antibiotics in any of these circumstances, because modest benefits should be weighted against risks. A strategy of "wait and see" (before prescribing) has been alternatively applied by clinicians, but the group of patients with delayed use of antibiotics in upper respiratory infection (including sore throat) might have more pain and malaise than those receiving immediate therapy [35]. The same is obviously true for the more severe infections, as meningitis [29]. The problem is that we do not have an equivalent clear number needed to treat for an adverse event (NNTR), mainly because the adverse events are more for the community (as antibiotic resistance) than for the individual patient. Moreover, how to compare the importance for the individual of these risks and benefits? A mild episode of nausea can be tolerated at the expenses of gaining a certain benefit in reduction of night cough in a patient with acute bronchitis? Certainly both the cultural and educational environments might modify the answer to this question.

30.15 THE PROBLEM OF PRESUMED MINIMAL BENEFITS THAT MIGHT BECOME SIGNIFICANT ONES

When a number of interventions that are expected to offer only marginal benefits unexpectedly result in a major benefit, we are confronted with this problem. This typically occurs when this major benefit is rarely associated to the intervention usually causing a small effect. Most prophylactic use of antibiotics is useless for the individual patients; nevertheless, if a life-threatening infection is effectively prevented, even in a minimal proportion of these patients, or if such use might serve to reduce the rate of cross-transmission between individuals, the prophylactic indication should be at least considered. Certainly the justification for the use of antibiotic therapy in acute tonsillitis is controversial in developed western societies, but certainly protects (80% protection) from acute rheumatic fever in low and middle income countries [2,32], and probably cuts the chain of transmission of Streptococcus pyogenes. Occasionally, this might also occur in developed nations. It seems clear that winter lower respiratory infections of presumed viral origin, should not be treated with antibiotics. Nevertheless, some studies have suggested that when community antibiotic prescribing is reduced beyond a certain limit under the aims of progressing in "rationality," mortality associated with community-acquired pneumonia might increase [30]. We might therefore envisage a number of "antibiotic abuses" (use of antibiotics not justified by its small benefit) that might have had a positive effect on human health. Similarly, the broad ("indiscriminate") use of antibiotics in upper respiratory tract infections might occasionally prevent severe infections, such as bacterial meningitis. Very

few data are available to document this possibility. If we compare the incidence (per 100,000 inhabitants) of confirmed cases of invasive meningococcal disease in five South European countries (France, Spain, Italy, Portugal, and Greece) with high antibiotic consumption in the community (on average 26.69 defined daily doses per 1000 inhabitants per day, or DDDs), with the same data of five North European countries (The Netherlands, England, Denmark, Norway, and Finland) with low antibiotic consumption (14.04 DDDs), a trend for higher mortality in the North (2.25 vs. 0.77 in the South) is suggested. Certainly other factors might have modulated this result, that is presented here only as an indication of the need of progressing in the analysis of this type of correlations.

30.16 THE DESIGN OF OBSERVATIONAL–ECOLOGICAL EXPERIMENTS TO DETERMINE ATTRIBUTABLE RISKS AND BENEFITS OF THE USE OF ANTIBIOTICS

The core of any observational study aiming to solve this problem can be summarized in following three points:

1. Establishment of appropriate cohorts of homogeneous individuals.
2. Continuous monitoring of any antibiotic uptake, by any reason, in these individuals.
3. Continuous monitoring of health progression and occurrence of any type of disease in the individuals.

The attributable risks or benefits of different levels of antibiotic consumption antibiotics are the fractions of the negative or positive outcomes that can be attributed to each particular level of antibiotic exposure. Rate fractions will be obtained by comparisons of the risks and benefits at each level of consumption for the same type of cohort [19,27], as it will be probably impossible to separate "cases" and "non-cases," as almost everybody take antibiotics if a sufficiently long time frame is considered. Such a way, differences should be based on cumulative analysis of quantities (How many times? For how long? What a dosage?), considering also the qualities (Which type of drug?). The complexity of the study is patent, as includes variable antibiotic exposures and variable responses. As we will analyze in the following paragraphs, the observable responses could be due to other factors than just the degree of antibiotic exposure (for instance, vaccination of hygiene). These factors should be taken into the consideration in the design of the study, as may certainly influence the determination of sampling principles and analytical methods for each type of observable event, as the specification of effect sizes or the statistical power needed. Bias is inevitable unless the heterogeneity of the events to be measured (for instance morbidity, or mortality) is incorporated in the analysis [38].

30.16.1 Facing Individual Variability: Blocking Strategies

One of the expected challenges of such type of experiments is the design of appropriate cohorting procedures. Natural variability in populations may swamp the observable effects, and the only way to proceed is by grouping experimental units of individuals with similar conditions.

- *Phenotypic variability*: Humans display along their life span a high number of phenotypes. The difference between a young and an old person is of phenotypic nature, both being "normal" for they age, but not equivalent either for drugs or microbes.
- *Pharmacological variability*: Physiologically based pharmacokinetic modeling procedures address the consideration of individual variability in the drug action with modulators such as physical condition, level of activity, hormonal status, or drug interactions [12].
- *Pathogenic variability*: The underlying illnesses increase the variability of the host for microbes or drugs. The acquisition of a pathogenic trait (e.g., diabetes, malnutrition, or immunosuppression) diversifies an otherwise homogeneous human population.
- *Behavioral variability*: There are important social, educational, and cultural influences in the attitudes of individuals towards antibiotic consumption.
- *Epidemiological variability*: The possibility of interacting with a given susceptible or resistant bacterial organism depends on the connectivity of the individual with other individuals, the frequency and epidemigenic value of the available microorganisms, the local prevalence of antibiotic resistance, and the hygienic measures, including vaccination.
- *Genotypic variability*: Inflammatory diseases and drug pharmacology are both influenced by genetic variability of the host. Common patterns in DNA sequence variation in the human genome will be available in the future and should help to refine the blocking process [23].

A number of techniques should be applied to address the methodological problems associated with variability: variance and covariance pilot studies, ANOVA, MANOVA, or Monte Carlo simulation. The definition and size of the cohorts, the effect size, and the required time for detecting events should be derived from these studies.

30.16.2 Facing the Heterogeneity of Antimicrobial Agents

Antimicrobial agents constitute a heterogeneous group of compounds. The analysis of the expected consequences of individual antibiotic consumption should consider such variety. For that purpose, it could be of interest to agree on a classification of antimicrobials in groups based on similarities of action. For instance, applying the utility maximization approach, we might assume that antibiotics of a particular group are effective (because of the risk of emergence or acquisition of resistance) only for a given finite number of

exposures [36], a number that can be different that that for members of another group.

30.16.3 Assumptions to be Tested and Possible Outcomes

In general, the assumptions to be tested about the influence of utilization of antibiotics for health are the following:

1. Appropriate utilization of antibiotics should be operatively considered as non-increasing risk activity, and eventually as a decreasing risk activity for the individual health.
2. Inappropriate utilization should be considered as an activity increasing the risks for individual health.

The complexity of the study might yield a complex type of response. For instance, it can be concluded that the benefits of a given level of increased uptake of antibiotics may be higher than the risks for particular groups of individuals, but the risks may overweight the benefits in other groups. The study may help to identify the variables influencing increased benefits and reducing risks in the different groups, and to help to design intervention studies.

30.16.4 Experiences in Other Fields

Scientists from other medical disciplines have carried out historical classic studies on the effect of environmental and behavioral factors on individual health, particularly in the field of detection of the increase in risks for cardiovascular disease. The Framingham Heart Study (http://www.framingham.com), with patients followed for more than 50 years, discovered the role of high cholesterol, HDL and PDL, high blood pressure, sedentary life, smoking, or diabetes in coronary artery disease, vascular disease, and stroke, and has produced more than 1000 scientific papers. The WHO sponsored MONICA (MONItoring CArdiovascular Disease) (http://www.ktl.fi/monica) included the study of risk factors in 170,000 people suffering from heart attack studied over a 10-year period in 37 countries. These types of studies may serve as a source of inspiration for new projects directed to estimate the risk and benefits of the use of antibiotics on the individual health, as our REBECCA Project [13].

30.17 CONCLUSION

The consumption of antimicrobial agents should be shaped accordingly to the utilitarian ethical principles. Utilitarianism sustains that an action or practice is right (when compared with any alternative action or practice) if it leads to the greatest possible balance of good consequences or to the least possible balance of bad consequences [14]. We believe that such philosophy should be mainly based in the individual convictions of consumers, appropriately based on the best data that we, scientists, are obliged to provide. Certainly a benefit for the community will necessarily follow in all aspects of public health, including ecological and evolutionary health [8].

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CHAPTER 31

Epidemic Diseases in the Past: History, Philosophy, and Religious Thought

D. Buchillet

Conditions et Territoires d'émergence des maladies, UR IRD 178, Paris, France

Epidemic diseases exerted a wide influence on human history through the ages. Some of them changed the course of civilizations: They brought devastation in lands affected, halted or exacerbated wars, defeated armies, indisposed leaders of nations, disrupted cities, destroyed or transformed state structures, sharpened political and social class divisions, and/or aggravated social antagonisms. Devastating populations that had never been in contact with them, they also enabled or facilitated European conquest, expansion, and colonization. But they also had cultural and intellectual effects, participating notably in the renewing of the medical understanding of the diseases. This chapter reviews briefly three of the major pestilences that swept over the world in the past at different times in repeated epidemic or pandemic waves (plague, smallpox, and cholera), examining their impact on human societies and the sociocultural and medical responses they gave rise to. They were chosen for their universal character, great virulence and rapidity of evolution, the high mortality rates they promoted in the past, and also because of the variety of social, cultural, and/or political responses they aroused.

31.1 PLAGUE[1]

Plague is an infectious disease caused by the gram-negative bacillus *Yersinia pestis* that ordinarily parasites some wild rodents (rabbits, marmots, ground squirrels, prairie dogs, etc.) without causing any effect and passes on from rodent to rodent through the bite of infected fleas. As a result of an ecological change or disturbance, some susceptible rodents such as the black or ship rat (*Rattus rattus*) or the brown or sewer rat (*Rattus norvegicus*) may enter in contact with the bacillus. Once infected, these develop an overwhelming infection and quickly die from it. Moreover, plague bacillus can accidentally be transmitted to humans through the bite of infected rat fleas (*Xenopsylla cheopis*) or of human fleas (*Pulex irritans*) that feed indifferently upon humans and rats. The bacillus is highly pathological for man and causes bubonic plague. In a few days, the majority of the infected present a kind of severe influenza and develop, on the fleabite site, a carbuncle or necrotic pustule. Within 6 days of the infection, the bacillus spreads to the regional lymph nodes that, because of inflammation, edema, and necrosis, transform themselves into big swollen and painful nodes (the buboes). About 60% of the untreated victims die within 10 days. Moreover, disease can be spread to other humans through the bite of infected human fleas [5,6,10,25]. In approximately 20% of cases, the bacillus reaches the lungs, causing the pneumonic plague that is spread directly from a person to another through the inhalation of infected droplets exhaled by victims while breathing, coughing, or spitting. Pneumonic plague is highly contagious and mortality rates are close to 100% [35]. In some cases, generally at the climax of the epidemic, the bacillus reaches the bloodstream causing a septicemia, which kills the victims in a few hours or days. It is the septicemic form [5,10,25].

Epidemics and/or pandemics of bubonic plague exploded in recurrent waves in the past, killing million people all over the

[1]The term "plague" was frequently used in the past as a synonym for pestilence, hence the difficulty to know when the disease plague first appeared in the world. In any case, historians now agree that the first detailed account of plague in its bubonic form is the description by the Byzantine historian Procopius of the Plague of Justinian, that swept over the world between the sixth and the eight centuries [5,6,10].

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

world. Some of them are famous for the ravages they have caused, as, for example, the plague of Justinian that swept over the world in recurrent waves during the sixth and the eight centuries. Originating in 541 in the Middle East or, possibly, Ethiopia [5,23], it reached in 542 Constantinople, governed by Emperor Justinian, hence the name "Justinian" attached to it. The plague also reached some parts of Western Europe (France, Italy, and Spain). The second pandemic plague known was called the "Black Death," probably because the skin of patients was covered with black plaques. Other names include "the Great Pestilence," "the Great Mortality," or "the Universal Plague" [24,25] because of the ravages it caused in humankind and the fact that it swept over the entire world. Originating in some region around the Caspian Sea, the Black Death spread to Central Asia, Constantinople, and the Mediterranean ports from where it invaded Western Europe. Lasting 7 years (1347–1354), it is said to have killed about 25% of the European population, for example, approximately 25 million people [6]. In the Muslim Middle East, one-third of the population died from the disease [35]. Given the rapidity of its dissemination into Europe and its high mortality rates, it seems that the bubonic and pneumonic forms of the disease were involved during this second pandemic, although their relative importance varied according to local conditions [25,35]. Progressing along the major maritime and land trade and communication routes, the disease stayed only a few months in each region or country, generally appearing in spring, summer and early autumn, and receding in winter [25]. After 1351, the Black Death began to retreat in Europe but bubonic plague continued to cause periodic, localized outbreaks (e.g., in Milan in 1639, in Genoa and Naples in 1656, in London in 1665, or in Marseille in 1720–1722, etc.) until the beginning of the eighteenth century.

Witnesses, chroniclers, and physicians noted certain peculiarities in the course of the Black Death. First, some regions (central Italy, southern France, East Anglia, and Scandinavia, etc.) suffered more than others whereas certain areas (as, e.g., Nuremberg) entirely escaped from it [6,25]. Second, although the disease spared no one, whatever its social origin, class or position, it seemed to affect with more severity the economically weaker population within a city or a region. Two reasons can be offered to this fact: People of privileged classes had the possibility to escape to more healthy regions when rumors of the epidemic began to circulate. Furthermore, when the causal agent of the disease as well as its mode of propagation were finally identified at the end of the nineteenth century,[2] this observation of a somewhat predilection for popular classes made sense considering their living conditions (crowded and unsanitary houses) that created a microenvironment favorable to the transmission of the disease from rats to humans and from person to person [6,25]. Third, persons whose occupations brought them into contact with the sick (medical doctors, hospital nurses, priests, notaries as well as the "ravens," as were called those who were in charge of the corpses removal and burials) and people who lived in communal institutions (members of religious orders, hospitalized persons, charity houses, for instance) also suffered greatly from the disease [5,6,24,25]. In numerous regions, famines probably worsened the ill effects of the disease, favoring its dissemination [6,23].

A third pandemic of plague, which spared most of Europe, being limited to some harbors, arose in central Asia around the mid-eighteenth century. From there, it spread to China (Canton in 1894, where it killed 100,000 persons) and India (Bombay in 1896, Calcutta in 1898, etc.). In Calcutta and in the province of Bengal, it is said to have killed 480,000 persons between 1898 and 1906 [24]. Australia, Eastern Africa, and North and South America were infected for the first time by the disease [10,24].

Between the end of the first pandemic (eight century) and the beginning of the second one (mid-fourteenth century), plague virtually disappeared from the Middle East and Europe. That is why, when the Black Death irrupted in Europe, it sounded as a new disease [6,24,25]. In front of its ravages, it rapidly caused panic and terror and generated diverse reactions, some of them extremely violent. Those who could afford it abandoned infected cities to more healthful regions, unwillingly acting as carriers of the bacillus and disseminating the disease. Others sought for spiritual remedies. Muslim and Christian religious authorities organized prayers, sessions of collective confession and penitence, processions, pilgrimages and special religious offices in order to supplicate God to put an end to the epidemic. Whereas for the most part of the Muslim world, the epidemic did not bear a moral connotation, for the Christians it was an unmistakable sign of the divine wrath because of human sins and, in some cases, as a sign of the approaching apocalypse [6,24,25]. Groups of flagellants were traveling across cities and public sessions of flagellations and of preaching repentance were organized in order to placate God's wrath [6,24,25].

In the Christian world, the epidemic also gave rise to violent reactions, resulting in the stigmatization and, sometimes, in massacres of entire communities used as scapegoats. In some European regions or countries (e.g., Aquitaine, Languedoc, Savoy, Provence, Catalonia, Aragon, Switzerland, southern Germany), Jews offered the conspicuous targets for accusations of plague spreading through poisoning local fountains, springs, and wells.[3] In some cases, these accusations were sustained by municipal or political authorities. Hundreds of Jewish communities were destroyed, their members exiled, imprisoned, tortured or burnt alive, and their property confiscated [6,24,25].

Whereas popular reactions sought for spiritual remedies and/or were involved in blaming and finding scapegoats,

[2]In 1894, A. Yersin identified in Hong Kong the bacillus in the tissues of plague-dead rats and of plague-dead humans. Two years later, P.S. Lewis demonstrated the link between the rat flea *Xenopsylla cheopis,* the rats and humans in the bubonic plague.

[3]It is interesting to note that during the plague epidemics in nineteenth century India, rumors of deliberate poisoning by doctors and hospital staff aroused among Indians [3].

medical authorities in Europe and the Middle East, basing themselves on Hippocrates and Galen's works who interpreted epidemics as a natural phenomenon resulting from the corruption of air and/or particular astrological events, recommended certain preventive (fortifying diet, rest, clean air, etc.) and curative (internal medication, salves, minor surgery, etc.) practices [25]. Moreover, certain governments and medical authorities of European countries, particularly in central and northern Italy, applied the existing sanitary legislation or instituted by law new large-scale public health and social measures in order to combat and prevent the plague: isolation of the sick, street cleaning, control of some odoriferous practices (as, e.g., butchery, tannery, dyeing), restrictions on travel to and from infected cities, control of the movement of infected goods, and so on. Public assemblies were prohibited by law, the burial of the dead regulated and medical doctors hired to treat the patients as many physicians had fled the cities like members of privileged classes. Boards of magistrates were in charge of the application of these measures [24,25]. Although largely ineffective given the complex ecology of the disease (it depends on the strict interaction of bacilli, rodents, fleas, and humans), these measures furnished, in some ways, the basis for widespread preventive practices such as quarantines, isolation of the sick and their household contacts, and sanitary cordons in succeeding epidemics in Europe [25]. Moreover, the decline of plague in Europe at the beginning of the eighteenth century is attributed by the historians to these public health measures and/or to a mutation of the bacillus in a less virulent strain [10]. In any case, these measures probably spared Europe from the third pandemic plague that aroused in Central Asia around the mid-eighteenth century.

31.2 SMALLPOX[4]

Differently to plague and, as we will see later, to cholera, and despite its horrible clinical manifestations and the high mortality rates it caused,[5] smallpox did not give rise to violent reactions among people. Also known as "the Great Fire" or the "Spotted Death" [18], an allusion to two of its more characteristic symptoms (the high "burning" fever and the rash, respectively), this acute eruptive disease is caused by an orthopoxvirus transmitted through droplet infection or also *via* cotton articles (clothing, shrouds, or blankets) contaminated with pus or scabs.[6] It is a strictly human virus with no known animal reservoir of disease. After an asymptomatic incubation period (1–3 weeks) and a brief period of invasion (3–4 days) commonly with high fever with a burning sensation, splitting headache, chills and nausea, and other symptoms, a diffuse rash appears. First on the face, it spreads to the trunk and then to the arms and legs: Papules evolve into vesicles with a head (3rd–4th day) and into pustules (5th–6th day). On the 8th or 9th day of the rash, the pustules begin to dry and scab. In some patients, the virus causes corneal ulceration, resulting in permanent blindness of the infected eye(s). Fatal in 20–50% of all the cases,[7] the survivors get a definitive immunity against the disease. In the past, smallpox often resulted in the permanent pockmarked and scarred face of those who recovered from it.

The geographic origin of the disease is unclear as are the dates when it first appeared into Europe and its routes of transmission. Nonetheless, certain archaeological evidences and ancient religious and medical writings seem to account for its antiquity in Asia and Egypt. For example, the *Susruta Samhita*, one of the oldest Sanskrit medical texts compiled before the fifth century, referred to a pustular disease named *masurika* which probably corresponds to smallpox. Moreover, as we will see later, the disease is associated to a goddess in India and is the object of an intense worship all over the country [2,3,18]. Scars resembling the typical pockmarks left by smallpox were found on the skin of three Egyptian mommies 3000 years old. In China, there is some evidence of the presence of smallpox in the fifth century. It seems that the disease was introduced in the north of the country during a war with the "barbarians" [18,20,21]. Under the Song dynasty (eleventh–thirteenth centuries), pediatricians published medical treatises on the disease, which seem to imply that smallpox was at that time a childhood disease among Chinese populations [20,21]. The *Xiao Er Yao Zheng Zhi Jue* [*Emergent Prescriptions for Pediatric Rash Diseases*] written in 1093 by the pediatrician Dong Ji is, for example, the first medical treatise on rash diseases in China. In this book, the author differentiated smallpox, measles, chickenpox, and scarlet fever [18,19,29,34]. Moreover, Chinese were the first in the world to use the variolation, a preventive method, which was intended to gain immunity in the individual through producing a controlled and mild form of the disease. The medical treatise *Yi Zong Jin Jian* [*Golden Mirror of Orthodox Medical Lineage*] written in 1742 by Wu Qian described four variolation techniques: water variolation (pulverized smallpox scabs were soaked into a moistened cotton swab which was inserted into the nostrils); dry variolation (powdered smallpox scabs were blown up into the nostrils *via* a long and

[4]The English term "small pox" is the translation of the French expression "la petite vérole" that was forged to distinguish its eruptive manifestations from those caused by syphilis known in France as "la grosse vérole" (the great pox) [18].

[5]It is considered by Hopkins [18], the author of a history of smallpox, as "The most terrible of all the ministers of death" in Europe. In the eighteenth century, it was twice more mortal than was plague in the seventeenth century, with case-fatality rates ranging from 8% to 10% against the 4% to 5% of the plague. At this period, it was considered a childhood disease [5,14].

[6]The smallpox virus can survive a few years outside human hosts in a dried state [31].

[7]Smallpox manifests itself in two forms: *Variola major,* the more virulent and lethal form, with a case-fatality rate ranging from 20% to 50% and *Variola minor* (*alastrim*), recognized in the late nineteenth century, with a mortality rate inferior to 1% and characterized by mild symptoms. Mortality rates of 100% were recorded during smallpox epidemic outbreaks among Native Americans [12,17,18].

fine silver tube); wet variolation (a cotton swab impregnated with pus from the smallpox vesicles was inserted into the nostrils); and variolation through clothing (a susceptible child was clothed for a few days with the undergarment of another one suffering from smallpox) [18,21,29,34]. It is worth noting that although water and dry variolation methods were devoid of danger for the variolated person because these methods used smallpox scabs of a child fully recovered from the disease, clothing and wet variolation methods could occasionally cause death because they were practiced with living matters [19,29,34]. Nevertheless, case–fatality rates were far lower in variolated peoples: About 1–2% died of smallpox against the 20 or 50% of those who contracted the disease naturally.

This preventive method was later introduced into the Americas and also into Europe after Lady Mary Wortley Montague (1689–1762), who learned about it when her husband was ambassador to Turkey, successfully used it on her own children. In Europe, variolation consisted in the cutaneous inoculation of pus or scab material from an infected patient into a susceptible person. The variolated would develop a mild form of the disease and be immune after recovery. Nevertheless, variolation was not easily or rapidly adopted by European and American populations. It was the matter of great debates between physicians because it was known to cause epidemics and/or fatal illness in some cases. By the end of the eighteenth century and in the beginning of the nineteenth century, it was progressively supplanted in Europe by Jennerian vaccination with cowpox virus [13,18,21,23].

In the Middle East, there is some proof of the existence of smallpox before the sixth century when a smallpox epidemic struck Ethiopian invaders threatening Mecca. During the tenth century, the Moslem physician Rhazes (al-Razi, 850–925) published a *Treatise on the Smallpox and Measles*, differentiating, for the first time in the history of the Western world, its symptoms from those of the measles, and revealing smallpox to be a common childhood disease in Southwest Asia at his time [13,18].

In Europe, there is no evidence of smallpox epidemics before the fourth century. When the Huns invaded France in 541, they beheaded on the doorstep of the Cathedral of Rheims the bishop who was said to have recovered from smallpox the year before. Because of this, he became after his canonization the patron saint of smallpox victims in Europe under the name of Saint Nicaise (Nicasius) [18]. In any case, although widespread, smallpox does not seem to have been among humanity's chief curses in Europe until the eighteenth century when it maintained high mortality rates, accounting for 10–15% of all deaths in some European countries, the majority of the victims being under 10 years of age [13,18]. Edward Jenner, the discoverer of the vaccination that replaced the variolation (or inoculation) method, estimated to 45,000 the annual number of victims of the disease in England. In European colonies in Asia and Africa, smallpox continued to claim for great numbers of victims until the early nineteenth century when immunization procedures were introduced [18]. The disease continued to rage in some countries of the Middle East until systematic vaccination campaigns, coordinated by World Health Organization (WHO), finally eradicated the disease. Between 1966 and 1977, national eradication campaigns (mass vaccinations, surveillance of smallpox outbreaks, etc.) were conducted by WHO in many countries and, in 1980, smallpox, one of the most dreadful and deadly diseases of humans, was officially declared as eradicated from the face of the world.

Unlike other epidemic diseases (e.g., cholera, as we will see later), smallpox attacked the rich and the poor alike. Contrarily to what happened with other pestilential diseases (e.g., plague and cholera) and despite its fearful and horrible clinical manifestations and the fact that it maintained high mortality rates in the seventeenth and eighteenth centuries, smallpox did not give rise to special popular reactions in Europe. It was considered a "familiar" or a childhood disease, being well integrated in the habits [14]. But in ancient Asia (especially in India, Bangladesh, China, and Japan) as well as in West Africa (Dahomey, in particular) and in Latin America (Brazil and Cuba) countries, the disease bore a strong religious signification. In India, where smallpox has long been endemic and was particularly raging under the British rule,[8] it occupied an important place in Hindu beliefs and rituals, being considered as a manifestation of the goddess *Sitala* (or *Shitala Mata*, literally, the "cool one" or "the chilly one," an allusion to the high fever and burning sensation she was supposed to relieve). Greatly feared, she was worshipped in virtually every part of the country, particularly by mothers on behalf of their children to protect them from smallpox, to ensure a mild attack or to secure the recovery of those ill with the disease. Shrines were built, temples erected, and people held propitiatory ceremonies to prevent the disease from circulating among the villages [2,3,18]. According to Hopkins [18], "Classically, and especially in Bengal, Shitala is represented by a woman riding an ass, with a broom in one hand (to sweep the disease along or to sweep away nonbelievers), a water-pot in her other arm (to hold the germs or to soothe feverish victims), a winnowing fan on her head (to sift the smallpox germs), and dressed in red clothes, sometimes with polka dots."[9] Cults to a goddess of smallpox were also reported in Bangladesh, China, and Japan.

For the Dahomeyan Yoruba (West Africa) as well as for the descendents of Yoruba who were brought to Brazil as slaves where they sought to maintain the cultural practices of their homeland, smallpox was attributed to an earth god named

[8]Accounting for several million deaths in the late nineteenth century alone, it was considered by British officials as "the scourge of India" [2,3].

[9]Interestingly, the variolation in India was considered as a religious ritual, involving a special dietary or ritual preparation, being performed by specific persons (the religious specialists *brahmins* in Bengal or the practitioners *tikadars* "mark-makers" in eastern India, for example) and accompanied by songs and prayers invoking the benevolence of the smallpox goddess. In contrast, vaccination, which was introduced by British medical officers in India at the beginning of the nineteenth century, did not bear a religious connotation [2,3].

Obaluaye (King of the Earth) in Yoruba land, *Omulu*, *Sapalá*, *Xapanã*, or *Obaluaê* in Afro-Brazilian religious cults (*candomblé* religion). Considered as the god of smallpox, he was known to punish people with the disease for wrong doing and also has the ability to cure it. Smallpox cults with offerings were organized to appease the smallpox deity in order to prevent the disease circulating among the communities [18,27]. In northeast Brazil, *Obaluaê* was identified with Christian saints as, for example, St. Roche, St. Sebastian, St. Lazarus, and St. Benoit [32, cited in 18] but, interestingly, not with St. Nicasius, the patron saint of smallpox in Europe [18].

In the sixteenth century, smallpox was unwillingly transported by Europeans into the New World still free from the disease, causing million victims among the indigenous populations.[10] In some cases, it was intentionally introduced through contaminated blankets among the Native Americans in order to resolve the "Indian problem" [11,30,33]. In many cases, as historical records showed, smallpox opened the way to European conquest and colonization of indigenous lands in the Americas, helping, for instance, Spanish colonists to knock down the pre-Colombian Aztec and Inca empires. European rapidly noticed an ethnic preference of the disease. A.F. Brandão, for instance, noted in his *Diálogos das Grandezas do Brasil* [*Dialogues on the Greatnesses of Brazil*] written in 1618 that "Fortunately, by the grace of God, smallpox affects only the natives, those who came from the African coast [African slaves] and the descendents of Whites and Indians that we name *mamelucos*. It was never transmitted to persons arriving from Portugal where they had been raised, be they of Portuguese or of any other European origin" [8].

The observation of a pathological selectivity of the disease (also made in other Latin American countries) was correct considering the fact that, at the time of the European conquest of the New World, smallpox was a childhood disease in Europe. Ignoring the causal agent of the disease, colonists and missionaries, who were assisting to the native demographic collapse, appealed to the only explanation they knew: that of the divine will. If epidemics were so catastrophic among the natives, it was, they thought, because of their moral and/or religious inferiority. The Christian God was pointing out the necessity of the spread of the Faith to all the natives and/or was punishing them for presumed crimes against nature (e.g., cannibalism) or for their simple reluctance to receive the Word [11,12]. Panic-stricken in front of this terrible and unknown scourge, Indians rapidly associated it with the arrival of the Whites and their goods into their territories. As some Jesuits noticed, they "die so easily that the bare look and smell of a Spaniard causes them to give up the ghost" [30, cited in 11; see also 12]. In any case, Native Americans quickly too perceived the pathological selectivity of the disease. Europeans were minimally affected whereas they were dying by millions. When Europeans caught the disease, it always had a benign course. Some indigenous groups felt betrayed by their gods or concluded that the Christian God possessed much greater power than their own [12,16]. Others saw the devastating epidemics as an expression of a "supernatural" or "magical" power of the Whites [4,22,23]. Still other ones, like the Desana of the Upper Rio Negro Region in Brazil, assimilated the White's apparent immunity to smallpox and other contagious diseases, along with their multiplicity, opulence and technical mastering to a special ontological characteristic: Their highly contagious nature which seemed to express itself only at the expense of the proper Indian existence and reality [9]. Whatever it may be, it seems evident that indigenous interpretations of contagious diseases cleared the way for European penetration and expansion and, in some cases, also precipitated Indian conversion to Christian faith.

31.3 CHOLERA

Cholera, also known as "epidemic cholera," "cholera *morbus*," "Asiatic cholera," or the "disease of the blue fear" (an allusion to the blue-grey skin of the patient, see [7]) is an acute debilitating diarrheal disease characterized by massive and incontrollable vomiting and diarrhea of "rice-water stools" [15] resulting in a severe dehydration and reducing the patient to an apathetic state with sunken eyes and a blue-grey skin. It is caused by the *Vibrio cholerae* bacterium, which is disseminated through the fecal–oral route as a consequence of sewage and fecal contamination of drinking water and food. It is now widely recognized that the disease was endemic in South Asia for at least 2000 years, especially in the delta lands of Ganges in West Bengal and Bangladesh, from where it has spread periodically in epidemic form to other parts of India and eventually of Asia [3,28]. In any case, there are some references on the disease in the *Susruta Samhita* medical treatise already mentioned. Hindu festivals and pilgrimages, which attract and concentrate pilgrims from endemic and nonendemic areas from all over India, have often been the scene of cholera epidemics in the past [3,28].

Although epidemic cholera appeared in Europe only at the beginning of the nineteenth century, Europeans knew of the disease through the descriptions of explorers, naturalists, and traders.[11] The Portuguese explorer Gaspar Correia, for

[10]Smallpox, measles, influenza, typhoid fever, bubonic plague, malaria, yellow fever are some of the diseases that were unleashed on New World indigenous societies without prior experience of them. Along with forced labor, wars of extermination, deportation, and so on, they were responsible for a demographic collapse among the Amerindians. This "pattern of bacteriological invasion" [1] was repeated in the eighteenth and nineteenth centuries among the Australian aborigines, the New Zealand Maori or the Pacific Islanders with the same lethal impact.

[11]There is some confusion in historical sources about the use of the term "cholera". In Western Europe and in the Americas, before the nineteenth century, the term cholera (or its synonyms "cholera *nostras*" or "sporadic cholera") designated endemic or sporadic diarrhea and historians agree that it was not caused by the vibrio. Today, the term cholera ("Asiatic cholera," "cholera *morbus,*" or "epidemic cholera") refers exclusively to the disease caused by the *Vibrio cholerae* [7,28].

example, reported the existence of "a disease, sudden-like, which struck with pain in the belly, so that a man did not last out eight hours time" [cited in 28]. The epidemic, which exploded in the spring of 1503 in the army of the Sovereign of Calicut, killed about 20,000 soldiers [28]. Other European reports repeatedly cited cholera epidemics in Goa in the 1600s, noting that the disease was more severe in June and July and that some epidemics were so devastating that people had no time to burry all the dead [28]. Garcia da Orta in his *Colloques des simples, des drogues et des choses médicinales de l'Inde*, first published in French in 1563, mentioned the presence in Goa of the cholera, highlighting its extreme virulence, the profound state of exhaustion it caused and the rapidity with which it killed an infected person. A "pestilential disorder" (probably a cholera-like disease) ravaged British troops in the Ganjam district of India in 1781 with 500 on a division of 5000 soldiers hospitalized. It reached Calcutta and other cities a few years later where it is said to have killed many persons and it continued to be reported in India during the rest of the eighteenth century and into the nineteenth century [3,28]. The virulence of the disease, which killed rapidly so many people, was frequently reported by European observers during the seventeenth and eighteenth centuries.

Although there are some discrepancies over the dates of the cholera pandemics (when they exactly began and ended), historians generally agree that, between the beginning of the nineteenth century and the second half of the twentieth century, cholera spread out of India, initiating a series of seven pandemic waves,[12] each one lasting a few years, that caused terror and panic and killed million persons all over the world [7,28]. The presence of English military troops and the augmentation of English commercial penetration in India and more largely in Asia are seen as the main factors that favored the dissemination of the disease out of the frontiers of the country [3,7,28]. Moreover, the intensification of commercial relations by navigation contributed to the multiplication of cholera epidemics during the nineteenth century.

The first of the pandemic waves, which initiated in the province of Bengal in 1817, had spread to other parts of India before reaching a few years later Ceylon, Burma, Siam (Bangkok), Malacca, Singapore, the Philippines, China, Java, Borneo, Persia, Egypt, the Caspian Sea shores, and Syria. It was during the second pandemic (about 1826–1837) that cholera reached for the first time Prussia, Poland, Hungary, Bulgaria, and Western Europe (England in October 1831, France in March 1832 where it killed about 100,000 persons). From Dublin, it crossed the Atlantic Ocean through an infected brig of emigrants and appeared in Quebec in June 1832. From there, it entered the United States. In 1833, it reached Spain, Portugal, the Caribbean and Latin America and in 1835, Italy. In Mexico, it is said to have killed approximately 15,000 persons in a few time [28]. During the third pandemic (about 1841–1859), the disease entered Afghanistan with the British troops, and then China, Persia, Central Asia, Europe, the Near East, North and South America, North Africa, producing the worst cholera years ever reported. It was during this pandemic that John Snow, in his study of the Broad Street water pump in London, demonstrated the transmission of the disease by contaminated water.

During the fourth pandemic (about 1863–1875) which reached, in 1865, the Middle and the Near East, about one-third of 90,000 pilgrims succumbed at Mecca. Those who survived brought the infection to Suez and to the Mediterranean ports from where it reached Western Europe and Russia where it killed many persons [7]. It was during the fifth pandemic (about 1881–1896) which reached the Mediterranean shores of Africa and Europe, Russia, Germany, North and Latin America, China, and Japan, that the German bacteriologist Robert Koch identified (1883–1884) in Calcutta and Alexandria the causative agent of the disease, thus initiating the modern understanding phase of the disease. During this pandemic, cholera installed itself in an endemic form in the countries localized in east and south-east of India (Ceylon, Indonesia, Java, Thailand, China, Japan, and Philippines). The sixth pandemic (about 1899–1923) followed much of the same routes than the preceding ones but did not reach Western Europe. Finally, the seventh pandemic, contrarily to the precedent ones, arose in Indonesia (in 1961) before reaching Bangladesh, India, Russia, and Africa a few years later. In 1991, it touched the port of Chancay in Peru. From there, it diffused rapidly along the 2000 km of the Peruvian coast and reached in 1992 the majority of the South (Bolivia, Ecuador, Colombia, Chile, Venezuela, Brazil) and Central (Mexico, Guatemala, Salvador, Honduras, Nicaragua) American countries. About 300,000 persons were infected in Peru, with a mortality of 1% [26]. At the same period, an epidemic outbreak killed more than 10,000 persons in Africa. This seven pandemic is always of actuality in some parts of the world [28].

It was reported that cholera did not affect evenly communities and populations, attacking with more severity persons of low conditions [7,15]. Human concentrations deriving from pilgrimages, markets, feasts, and so on, also appeared to furnish the ideal conditions for the explosion of an epidemic outburst, as it was frequently reported in the past in India [3,28]. Although the disease was essentially imputed by medical authorities to some environmental conditions (weather, seasons, bad air and miasmas, dietary transgressions), the question of its contagiousness was a matter of debate between contagionists and infectionists. The identification by Koch of the causative agent of epidemic cholera (the vibrio) and the demonstration by Snow of the water-borne nature of the disease furnished the base for the recognition of the impact of socioeconomic and sanitary conditions on health. These two discoveries also proved the contagious character of the disease [15,28].

[12]The first six pandemics are attributed to the *V. cholerae* biotype 01. The seventh pandemic which, contrarily to the precedent ones, arose in Indonesia, was attributed to the *V. cholerae* 01 biotype El Tor serotype Inaba. Recent epidemiological works seem to account for an influence of the *El Niño* climatic phenomenon on the multiplication of the vibrio [26].

The 1832 cholera epidemic in Paris is particularly illustrative of the different social and political meanings that can be attached to a same epidemic event by different groups in a society. First, in France as well as in other European countries, cholera mortality was higher among popular classes than it was among upper classes that always had the possibility to flee to cholera-free regions, as we have seen in the case of the Black Death pandemic. Second, the rapidity of the evolution of the disease (it was said to kill a healthy person in a few hours) as well as the fact that it struck suddenly its victims in the more diverse settings (at home, in hospitals, in streets, in public places, in taverns after eating or drinking or in places frequented by beggars and unemployed, etc.) exalted popular imagination. To ordinary persons, some of its symptoms (massive and violent diarrhea, vomiting, spasms, and convulsions) look like the symptoms of poisoning. The fact that it can strike its victims after eating or drinking and the rapidity with which it killed also lent credence to the rumor of poisoning [15].

All over Europe, popular reactions were dominated by panic, fear of poisoning, and anger. Because the disease was first flourishing among the poor, these pointed out the same enemies. The cholera epidemic was a consequence of a plot hatched by external enemies (privileged classes, members of government and also medical doctors who were seen as their allies) to poison the poor by throwing poisons in local springs, wells, food, and drinks. Some imagined that it was in order to release their body for anatomical experiences on behalf of the rich, others that government agents tried to avert an imminent famine, reduce the burden of poverty by ruling out its main victims, resolve the problem of unemployment by eliminating unproductive elements of the society, or also to get rid of dissidents [7,15]. Popular classes reacted violently. Rebellions and destruction of property took place. Suspects of poisoning were arrested, beaten, or massacred by the furious populace.

For their part, when rumors of cholera began to circulate, many members of the privileged classes tried to escape from the disease by fleeing to more healthy regions. When the disease invaded the rich districts of the city and they also began to fall ill and die from it, albeit in smaller numbers than members of the working classes, they attributed the contagion to a corrupted air arriving from the popular and impoverished districts of the city [15]. The rapid progression of the disease in the entire society and the high mortality rates registered in popular classes, they thought, was a consequence of the "barbarousness" and "moral inferiority" of the poor. These were blamed as being incubators and disseminators of the disease, a threat to public welfare and fomenters of riots [15]. The 1832 cholera epidemic, which killed 20,000 persons only in Paris, acted in this way as a revealer of social and political hate and class antagonisms.[13]

[13]See [3] for another example of the diversity of meanings that were attached to cholera epidemics in India under the British rule.

31.4 CONCLUSION

In the past and more recently, as the advent of AIDS in the 1980s reminds us, epidemic diseases take on a wider significance, serving to prove, undermine and/or reshape religious, social and political ideas and attitudes. Their dissemination and impact on human populations were often influenced by social and political factors. Moreover, they can affect different groups in a society in varying ways and degrees. Different and sometimes divergent meanings can also be attached to a same epidemic event, as we saw for the 1832 cholera epidemic in Paris. In some cases, epidemic diseases contributed to the renewing of official medical thought and practice. Social and public-health measures applied in some European countries during the Black Death years, although largely ineffective given the natural history of the disease, nonetheless furnished the basis for public-health policies for subsequent epidemics. The 1832 cholera epidemic in Paris, and more generally in France, revealed the impact of living conditions and sanitation on health, contributing to the development of private hygiene and public sanitation and to the reinforcing of the medical power. These founding events like many others, as for example, the discovery of the causative agents of the diseases, caused a revolution in public health and medicine that lead to the dream of the eradication of the infectious diseases all over the world. The advent of AIDS as well as the recent emergence of new or previously unrecognized virus and/or the resurgence of infectious diseases once thought to be controlled, put unfortunately an end to this optimism in countries complacent with their success in banishing infectious diseases worldwide.

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CHAPTER 32

Fundamentals, Domains, and Diffusion of Disease Emergence: Tools and Strategies for a New Paradigm

Jean-Paul J. Gonzalez,[1,2] Philippe Barbazan,[1,2] François Baillon,[3,4] Julien Capelle,[5] Damien Chevallier,[3,4] Jean-Paul Cornet,[1,2] Florence Fournet,[3] Vincent Herbreteau,[1,2] Jean-Pierre Hugot,[6,2] Meriadeg Le Gouilh,[1,2,7] Eric Leroy,[8] Bernard Mondet,[9] Narong Nitatpattana,[10] Stephane Rican,[11] Gérard Salem,[11,12] Wailarut Tuntrapasarat,[13] and Marc Souris[1,2]

[1]*IRD Unit 178 "Conditions et territoires d'émergence des maladies," Paris, France*

[2]*Unit 178 IRD, Faculty of Sciences, Mahidol University, Bangkok, Thailand*

[3]*Unit 178 IRD, Ouagadougou, Burkina Faso*

[4]*IRD Unit 176 "Aires protégées," IRD Orléans, France*

[5]*Lyon Veterinary School, Lyon, France Unit 178 IRD*

[6]*Museum National d'Histoire Naturelle, Paris, France*

[7]*Fondation Carrefour, Paris*

[8]*Centre International de Recherche Médicale de Franceville, Gabon*

[9]*Institut Français de Pondichéry, Pondicherry, India*

[10]*Center for Vaccine Development, Mahidol University, Bangkok, Thailand*

[11]*Institut du Cancer, Paris, France*

[12]*Université de Paris X, Paris-Nanterre*

[13]*Faculty of Public Health Mahidol University TropMed, Thailand*

FOREWORD

In this chapter, we will focus on establishing the origin of infectious diseases. Taken that disease is an entity strictly dependent on human thought, it will not deal with the origin of a parasite *sensu lato* but with its intrinsic pathogenicity, it will not be about the animal's role in the natural cycle of a parasite but about the origin of their role as host, reservoir or vector of a pathogen and how humans, as individuals or societies, witness, face, and understand a disease when it appears in our bodies, or spreads through a community.

The cognitive approach to infectious disease is studied to enable better understanding of fundamentals of disease emergence mechanisms and definition of borders around domain expansion.

The concept of "Disease Emergence" constitutes in itself the epicenter of this study, which will not be focused on an object—a disease-centered approach—but a systemic approach to disease, among other diseases, in a given environment and time scale. It will not be purely a study of epidemiology, spatial or descriptive, but a wide integrative approach centered on the understanding of all mechanisms of

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emergence and encompassing them from molecular to global level.

We present scientific thought about the nature of infectious disease not only in nature but also in the collective consciousness with as final goal the understanding of the rise and fall of plagues threatening all biological life forms on earth.

In order to present the entire concept of disease emergence, its understanding and offensive strategies to counterattack germs, we will therefore discuss the following aspects:

- Concepts and the "domino effect" of disease emergence: Understanding the mechanisms involved from the index cases to a pandemic;
- Strategies to address the challenge, and tools to investigate: Tools and methods developed to study the dynamics of the emergence;
- Some explanations and solutions that are shared by several exemplary diseases: Diseases with a profile of generic evolution applicable to a common nosology and diseases with emergence and single diffusion dynamics.

32.1 FROM NOSOLOGY TO CONCEPT

From "emerging diseases" to "fundamentals of disease emergence": Two propositions, succeeding each other, show during the past two decades, an evolution of thought which went from the nosological, objective view point of "emerging diseases" to a more conceptual and subjective approach that included factors and mechanisms of emergence as "fundamentals of disease emergence." Later on, spatial and social dimensions were added with the term "domains of emergence."[1] A new more exhaustive way of thinking evolved, with a temporal dimension, which necessitated a multidisciplinary approach.

32.1.1 Emerging Diseases

32.1.1.1 Change in awareness The scientific community decided in the late 1980s to forge a new semiological concept encompassing arboviruses, that is, vector-borne transmitted diseases, such as hemorrhagic fevers and other apparently new diseases.

32.1.1.2 An old concept However, the phenomenon of disease emergence was understood much earlier by scientists paving the way to a more general concept focusing on the life and death of diseases and their epidemic occurrences.

In fact the concept preexisted and was clearly expressed by Charles Nicolle,[2] director of the Institute Pasteur de Tunis, in 1920 when he gave a talk at the university entitled "Life and death of Infectious Diseases" presenting the idea of an intrinsic one dynamic approach for infectious diseases and moreover a sense of the rare, but no less important, vanishing diseases.

Charles Nicolle developed in his book "*Le Destin des maladies infectieuses*" [50] his vision on the "*Naissance, vie et mort des maladies infectieuses*." Later, in the 1970s, Max Germain and his colleagues [18] described the concept of "zone of emergence of Yellow Fever" in west and central Africa, pointing out the necessity of a convergence of several factors (i.e., primate herd immunity, seasonal vector activity) to allow the emergence of the disease in a given environment (i.e., the sub-Sudanese phytogeographic domain). Since then, both concepts of emerging diseases and disease emergence were used as a practical approach to focus attention and research on new pathogens and disease evolution in a changing world.

Over time, the concept of emerging diseases appeared gradually within the medical scientific community during the late 1980s when viral hemorrhagic fevers (e.g., Lassa fever, Ebola fever) or other surprisingly severe syndromes (e.g., Lyme disease, Legionella diseases) were identified and large and sudden epidemics were witnessed (e.g., HIV). It was only in 1987 that Joshua Lederberg, Robert B. Shope, and Mary Wilson formally coined the term of emerging and reemerging diseases. Responding to political demand they addressed the issue of new infectious diseases around for the last 30 years. What was truth or artifact? What measures should be taken? [38]

Since then, national and international meetings periodically arise, books, publications, journals are produced, university and institutional departments and laboratories are formed with teams of specialists that focus on emerging diseases with the objective of preventing or forecasting epidemics. But, after deploying scientific potential, mostly to fulfill political and public demand, the main scientific objective lies in understanding the emergence of new pathogens, new diseases, and their reemergence in virgin populations or domains after a long epidemic silence.

In the 1990's, most of the researchers in arbovirology already involved in the study of deadly hemorrhagic fever of viral origin joined the current movement in medicine by focusing their study field on the *emerging* diseases. At the same time, WHO created a special force on emerging diseases, at the Centre for Disease Control and Prevention (CDC) in Atlanta (Georgia, USA); the "special pathogens branch" became the cradle of the emerging disease team, and also, several laboratories across the world were fully or partially oriented on such research.

As for a research unit example, our IRD team named "fundamentals and domains of disease emergence" as developed in 1997 the first "Research Centre for Emerging Diseases,"

[1] *In French "territoires d'émergence"* a term for designing a specific domain with borders of natural, human, biological origin where the event of emergence happened.

[2] Charles Nicolle 1866–1936 (Nobel prize of Medicine, 1928): Doctor, biologist, born in 1866 in Rouen (France), died in Tunis in 1936 in Tunis (Tunisia). Doctor of the hospitals of Rouen, Director of the Pasteur Institute of Tunis (1902–1936), discovered the role of the louse in the exanthematic transmission of typhus (1909) "the pathocenosis, a new historical, epidemiologic and clinical approach to diseases."

hosted by Mahidol University in Thailand. At the beginning of the twenty-first century, we merged human and social sciences, computer sciences, and specialized biomedicine. Health geography, health information systems, and health ecology have been combined, thus adopting a transdisciplinary approach toward understanding the emergence of diseases. Today, above all concepts, health security, biosecurity, and its corollary biodefense appears as a most important concept showing the dependence between social and environmental factors underlying the fundamentals of disease emergence.

A TYPOLOGY OF EMERGING INFECTIOUS DISEASES (EID)

(1) Emergence of an unedited infectious disease (unknown newly described pathogen, new clinical picture, and a known pathogen)
(2) Reemergence, after a prolonged period of silence, of a previously known infectious disease
(3) Spread of a known infectious disease among new territories and/or virgin populations

32.1.2 Understanding the Fundamentals of Emergence

In this chapter the phenomenon of disease emergence is tackled from three angles, as a multidisciplinary approach is required, it focuses on

- the old framework of the emergent diseases;
- the historical concept of pathocenosis which deals with emergence in time and space;
- health ecology as opposed to traditional epidemiology, which is generally restrained to a more linear disease analysis.

32.1.2.1 Emerging diseases have specific timeframe

All diseases one day emerge, either in the biosphere or in the collective conscience, but the reactions are the same; to fight, control, and contain them. The concept of emergence in its present meaning makes it possible to focus research on factors of emergence, to fight and protect against disease, and using emergence indicators, to prevent and control them. The emergent, new viral diseases (new virus, new syndrome), reemerging (nonimmune populations or interepidemic silence), or expanding (due to infected hosts and vectors traveling to unaffected areas), are complex and result from host–vector–virus interactions in often cryptic natural cycles, for which the environment plays a major role.

Diseases are generally regrouped into nosological frameworks to develop comprehensive studies (e.g., arbovirosis, viral encephalitis, water-borne diseases). The EID concept was chosen to help identify common risk factors, diffusion agents, and mechanisms of increasing or changing pathogenicity. Prevalent EID and later ED were categorized and a typology defined regarding risk and indicators of risk, as for example, risk of natural origin, risk associated with human behavior, and risk linked to environment.

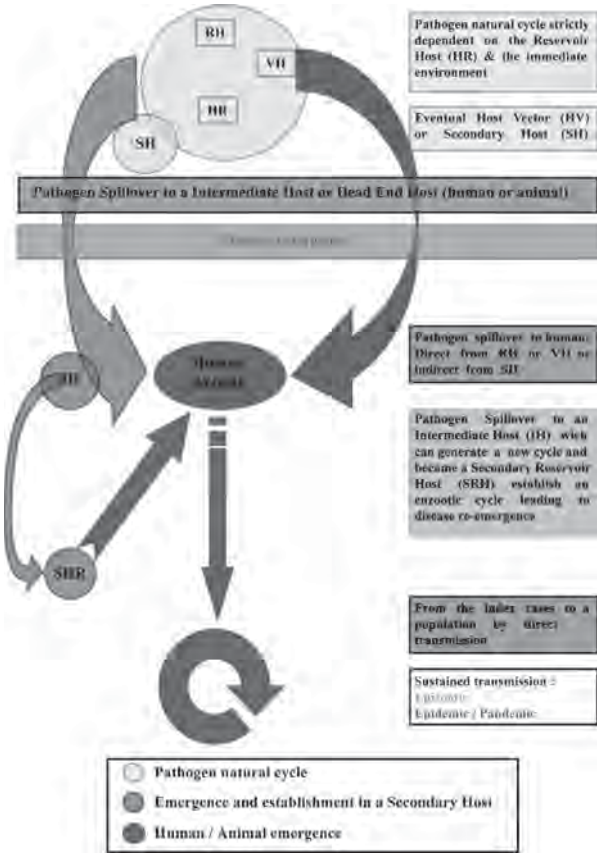

Fig. 32.1. Infectious disease emergence (a sketch): the general pathways of infectious disease emergence (adapted from Childs et al., 2004, in press): (I) From a natural cryptic cycle of a pathogen including a reservoir host (RH), eventually a vector host (VH) or a secondary host (SH) between reservoir specimens and a pathogen, a contact and a spillover are necessary (under specific mechanisms including host behavior, pathogen changes, and environmental factors) and in sufficient quantity to establish the emergence of a pathogen in a naive host (human or animal index cases). (II) RH and SH will favor the transmission to other species including wild animals (proximity to natural cycle), domestic animals, and humans. Humans as SH can also be infected by animals and/or eventual secondary vectors . (III) Population density will affect behavior of susceptible hosts; biodiversity will favor a sustained transmission and spread of diseases (parasite fitness, anthropogenic influences, SH adaptation to the pathogen, interconnectivity, transport; surveillance and control). (IV) Emergence of epidemics to a pandemic situation will require sustained intrasusceptible host or SH transmission of the pathogen. Also, an adaptation within the SH can modify genetically the pathogens with a novel phenotype favoring the spread of disease. (V) "However pathogens may fail to initiate cross-species infection following exposure or fail to generate secondary infections both cases interrupt the emergence process." See color plates.

TABLE 32.1. Domains and Emergence Conditions of Some Recent Exemplary Human Diseases

Year of Emergence	Emerging Disease	Pathogenic Agent	Place and Domain of Emergence	Factors of Emergence	
				Main Probable Factor	Secondary Factor
Viruses					
1958	Argentine hemorrhagic fever (AHF)	Junin virus (arenavirus)	Argentina; Pampa, corn fields	Changes in agricultural practices of corn harvest (maize mechanical harvesting)	Conditions favoring rodent host/vector pullulation and increasing human contact
1981	Acute immuno-deficiency syndrome (AIDS)	Human immunodeficiency virus HIV (HIV 1 and 2; retroviruses)	USA; urban communities	Yet not entirely understood origin of the virus introduction; sexual contact with or exposure to blood or tissues of an infected person	Changes in lifestyles; Increasing international travel; multiple sexual partners; increased intravenous drug use addiction; vertical transmission; invasive medical tecnology (transfusion; transplants)
1959	Bolivian hemhorragic fever (BHF)	Machupo virus (arenavirus)	Bolivia; Beni Province, in house	Population increase of infected rats gathering for food	Inscreasing peridomestic contacts
1986	Bovine spongiform encephalopathy (BSE) in cows	BSE agent (prion)	Great Britain; cattle rising aera	Feeding cattle with products containing prion infected sheep tissue	Changes in the rendering process
2005	Chikungunya arthritis	Chikungunya virus (alphavirus)	Indian Ocean Islands	Introduction of the virus in the Islands (Comore, Reunion) by a yet to be discover mean (plane, wind?)	Pullulation of infected competent mosquito, lack of control
1996	Creutzfel Jacob disease	CJD agent (prion)	Great Britain	Emergence of a BSE variant	Etiology recognition and virus variant identification
1983	Crimean-Congo hemorrhagic fever	CCHF virus	West Africa Sahel – Senegal river basin	Ecological changes favoring increased human exposure to ticks on sheep and small wild animals	Bite of an infected adult tick
1953	Dengue hemorrhagic fever (DHF)	Dengue virus 1,2,3,4	Philippines, Thailand (1956)	Increasing human population density in cities and favoring vector breeding sites (water storage) (*Aedes aegypti*)	Repeted infections by Dengue serotypes (1,2,3,4); high circulation of strains from various geographical origin
1976	Ebola hemorrhagic fever	Ebola virus types Zaire and Sudan	Zaire and Sudan	Congolese rains forest penetraiton by humans	Close contact with infected game (hunting) and/or with the host reservoir (bats) infected biological products; nosocomial transmission, needle spread
1976	Hemorrhagic fever with renal syndrome (HFRS)	Hantaan virus (Hantavirus)	Korea	Exposure of military US troups to rodent infected habitat during the armed conflict in Korea	Close contact with rodent infected specimen and/or habitat
1993	Hantavirus pulmonary syndrome (HPS)	Sin Nombre Virus (Hantavirus)	USA, "Four corners" area	Human invasion of virus ecologic niche by rice field agriculture; close contact with the infected rodent natural reservoir; inhalation of aerosolized rodent urine and feces	Climatic condition favoring a rodent pullulation

1997	Higly pathogenic avian influenza (HPAI)	HPAI H5N1 virus (Paramyxovirus)	Hong Kong; chicken farms of South East Asia	Animal–animal Influenza virus reassortment; emergence H5N1 avian influenza virus reassortant; extensive chicken farming	Integrated pig–duck agriculture; close contact with infected chicken
1889, 1890, 1918, 1957, 1968	Influenza pandemic	Influenza A virus (Paramyxovirus)	Russia, India, USA, China, HongKong	Animal–human virus reassortment and antigenic shift	Airbone (crowed, enclosed spaces)
1999	Japanese encephalitis (JE)	JE virus (flavivirus)	Australia, North Quensland Tip	Torres detroit passagge by air borne infected mosquitoes (wind effect?)	Concurrent large epidemic and epizootic in Indonesia
1997	Japanese encephalitis	JE virus (flavivirus)	Japan	Changing agricultural practices, extensive pig farming	Bite of an infective mosquito
1969	Lassa fever	Lassa virus (arenavirus)	Nigeria; rural area	Hospital exposure to index case—Rodent exposure	Rat vector reservoir pullulation in houses
1956	Marburg disease	Marburg virus (filovirus)	Germany; monkey experimental laboratories	Trade an use of wild monkeys imported from Africa—use of organs for scientific purpose	Preparing monkey cell kidney for medical research; direct contact with infected tissue
1961	Oropuche fever	Oropouche virus (Bunyavirus)	Brazil Amazonia Cacao plantations	Developing and incrinsing agriculture	Cacao hulls are breeding sites for the culicoides arthropod vector
1987	Rift Valley fever (RVF)	RVF virus (bunyavirus)	Mauritania, cattle rising area	Dramatic increase of mosquito vector breeding sites by filing a new dam; weather (rainfall) and cattle migration guided by artificial water holes	Drought and hight density of herds and humans around water wells. importation of infected mosquitoes and/or animals; development (dams, irrigation)
1957 and 1986	Seoul infection	Seoul virus (hantavirus)	Korea, USA harbors	Increasing population of urban rats	Spread of rat host trough commercial ships
2003	Severe acute respiratory syndrom (SARS)	SARS Coronavirus	South Province of China	Eating practices of infected wild animals (viverrids)	Catching and preparing infected Civetta
17th and 18th centuries	Smallpox	Smallpox virus (poxvirus)	North American colonies	A new virus arrive with infected European traveling to the Americas	Nonimmune population
1965	Yellow fever	Yellow fever virus (flavivirus)	Nigeria	Reintroduced from sylvan source by viremic travellers; lack of immunization campaing	Mosquito exposure without immunization
1968	Viral Gastroenteritis	Norwalk & -like virus (Norovirus)	USA	Increased recognition	Most likely fecal–oral; drinking and swimming water, and uncooked foods

(Continued)

TABLE 32.1. (Continued)

Year of Emergence	Emerging Disease	Pathogenic Agent	Place and Domain of Emergence	Factors of Emergence	
				Main Probable Factor	Secondary Factor
Bacteria					
1975	Babesiosis fever (malarialike infection)	*Babesia microti*	USA; Long Island, New York	Reforestation; deer population *(Babesia* tick vector host) increases, increasing outdoor recreational activity	Bite of an infected Ixodes tick feeding on mice and deers
1980	Campylobacter gastroenteritis	*Campylobacter jejuni*	Sweden	Ingestion of contaminated food, water, or milk; fecal–oral spread from infected person or animal	Increased recognition consumption of uncooked poultry
1986	Human monocytic erlichiosis	Ehrlichia chaffeensis	USA	Unknown; tick is suspected vector	Increased recognition; possibly increase in host and vector populations
1996	Hemorrhagic colitis	*Escherichia coli* O157:H7	USA	Ingestion of contaminated food, undercooked beef and raw milk	Likely due to the development of a new pathogen
1976	Legionnaires' disease	*Legionella pneumophila*	USA, Pennsylvania	Air-cooling systems, water supplies	Recognition in an epidemic situation
1975	Lyme disease	*Borrelia burgdorferi*	USA, Lyme Connecticut	Bite of infective *Ixodes* tick (deer ticks)	Increase in deer and human populations in wooded areas
Parasites					
1976	Malaria	*Plasmodium*	92 Countries of Africa	Global eradication policy abandoned, economics, growing interchange of populations	Drug and insecticide resistance, political unrest, lack of public health, human behavior, poverty
1960	Anisakiasis: acute localized enteritis	*Ansiakis marina* A. simplex	Netherland	Increasing consumption of crude fish	Recognized in Japan since 1955
1970	Gnathostomiasis	*Gnathostoma spinigerum,* G. *hispidum*	South America	Increasing consumption of crude fish (sashimi or "ceviche"); endemic in Asia	Undercooked fish or poultry, drinking water with infected Cyclops
1994	Acute pneumocystis pneumonia	*Pneumocystis carinii*	USA, Europe	Immunosuppressed patients, HIV infected patients CD4+ cell count	Unknown; possibly reactivation of latent infection

Disease is a human concept all "new" diseases will one day emerge in a given population and environment. This table cannot therefore be exhaustive, the diseases selected were chosen for their exemplary nature and belonging to a particular period of time, population or original geography with complex, or rare transmission.

The concept of emerging diseases was developed in order to adopt a specific strategy of control and prevention in response to the sudden appearance of unedited pathogens and diseases during the last century, the rapid propagation of such diseases at a regional or global level, and the challenge for public health officers to counterattack such a growing threat in ever changing environments and societies.

In the context of emergent diseases, new diseases or emergence of the diseases, several remarkable works have been written, which deal with infection risk at all levels [12, 38, 47, 56, 59].

The emergent and or new viral diseases (new virus, new syndrome), reemergent (nonimmune population or inter epidemic silence), or expanding (from hosts and infected vectors traveling) are among the most important and represent two-third of the present complex. There are the results of host–vector–virus interactions in natural cycles often cryptic for which the environment plays a major role. They are generally transmissible zoonosis, often by a vector and, frequently infections of exceptional gravity (encephalitis, hemorrhagic fever) or of exacerbated epidemic kind (AIDS, SARS, influenza). The dynamic of these diseases are subject to constantly changing, human environments and genomic plasticity of evolving viruses.

32.1.2.2 Toward the historical concept of pathocenosis

The concept of pathocenosis is presented like an element structuring disease emergence clearly identified as a temporal (emergence *sensu stricto*) and spatial (the territory of the disease) phenomenon. The term pathocenosis, created by Mirko Grmek, historian of biomedical sciences is not only a neologism, which so elegantly associates "pathology" and "biocenosis" but above all a concept to define "pathological states within a population determined in time and space." "However, Mr. Grmek's idea is not limited to describing these pathological states. It (also) postulates that the frequency and distribution of each disease depends on the frequency and distribution of all the other diseases. Examples of interdependences between pathological states, whether in the case of a synergy or an antagonism, are numerous."[3]

The pathocenosis naturally tends to equilibrium, and is therefore particularly sensitive (observable) in a stable ecological situation. It is known that the human immune-deficiency virus (HIV) by its immune-suppressor effect will favor patient infection by Koch bacillus and tuberculosis will then become the syndrome dominating the patient. *Escherichia coli*, a commensal bacterium of the digestive tract of all humans, can, under certain conditions, changes its phenotype to increase its invasive potential, weakening intestinal epithelium, paving the way of intense replication of latent viruses such as are Rotavirus or picornaviruses becoming enteropathogen.

PATHOCENOSIS is a state, which tends toward balance but exists in perpetual imbalance. This creates the conditions of disease emergence; rebuilding the pathocenosis toward a nonattainable balance and which with this new more or less significant imbalance will integrate or eliminate pathology.

As with pathocenosis, disease research strategy, aims to understand the disease emergence mechanisms and imposes a multidisciplinary approach; historical, epidemiological, biological, clinical, and environmental. Historians, health geographers, philologists, epidemiologists, mathematicians, or even specialists of emergent diseases find themselves in the same dynamic flow of thought, that of the concept of disease emergence like an imbalance of pathocenosis in a place, a population, and a given time. Specialists in these various disciplines met to compare and share their knowledge in a 3-day seminar at the Abbey of Ardennes in Caen in April 2005.[4]

Thus, pathocenosis globally tends toward a balance observed in stable situations but can undergo brutal changes, which favor the emergence of new diseases or of known ones that, will reemerge after an often incompletely understood inter epidemic silence.

For any given population, one will see over time the domains of diseases, coexisting, succeeding, and gradually being replaced by others. The causes of these effects are multiple; they interact to produce a complex epidemiologic profile with a certain stability leading to a silence or an evolution before a renewed epidemic eruption when appropriate conditions are finally installed.

Lastly, Gerard Lambert, writes[5] "(. . .) the passage of a pathocenosis dominated by infectious diseases to a pathocenosis in which the degenerative diseases (in particular cardiovascular diseases and cancer) take the 'lion share,' constitutes the last great rupture of the history" (. . .) and further "(. . .) the relevance with which Mirko Grmek had at the time analyzed the origins of AIDS pandemic [22] in comparison with the pathocenosis, proves the potential operational value of this concept (. . .)."

32.1.2.3 Health ecology

In a given ecosystem the emergence of infectious diseases depends on dynamics of human societies, of animal populations and also of the germ's

[3] Gerard Lambert, 2005, personal communication.

[4] Days of study where organized by the Workshop on the pathocenosis in collaboration with Louise L. Lambrichs and in partnership with the Institut Mémoires de l'édition contemporaine (IMEC), at the abbey of Ardennes (Caen) April 13-16, 2005.

[5] Personal communication, 2005.

pathogens, factors which are all more or less dependant on the varying environments at both micro (genetics) and macro scale (populations and land).

32.1.2.3.1 Structural change of human society. A rapidly growing global population changing societies, a tremendous increase of communications and exchanges of all kinds, an evolving environment under human and natural influences, and the potential globalization of any local phenomenon have characterized the past century. When it comes to health, besides the outstanding technological and fundamental progress in medical science over the past 50 years, the inequality between societies from urban to rural, from industrialized to developing countries, are a permanent challenge for health carers, officers, and politicians. Health care access underlines the disparities between the two extremes; despite the efficient and thoroughly conducted immunization programs and campaigns, the cost is unbalanced between control and prevention. Medications are increasing in price, as are public health systems, which must deal with the specific challenge of unprepared populations facing the emergence of unedited diseases.

32.1.2.3.2 Changing societies: Transition from Rural to the Urban Society. Let us consider the factors that have influenced the infectious risk increase: This century is without precedent in terms of demographic growth, human exchanges, transport of domestic animals from one continent to another, the transformation of landscapes (deforestation, land development), and anarchistic use of modern tools (antibiotics, pesticides, new foods). Pandemics are fast and frequent, the germs and their vectors escape weapons of prevention and control, the clinical picture changes under the combined effect of emergent pathogenic agents (virus of AIDS) and the new therapeutic treatments (immune-therapy). The biodiversity of the pathogenic germs, globalization of human behavior, global warming, early alarm systems, strategies of biodefense are the many concepts that lead to the redefinition of health management.

32.1.2.3.3 Hosts, vectors, reservoirs This trilogy, considered formerly as the dogma defining infectious diseases as a whole, is recomposed today around essential elements of modern medical thought: It is no longer the role of the individual, but his behavior and actions on the environment (including social environment); that prescribes the necessary steps to be taken for the improvement of health care.

32.1.2.3.4 Parasites The parasites *sensu lato* are dependant on the hosts who carry them and constitute the microenvironment of their existence. These parasites will then not only be dependent on their host but also indirectly on the environment, which modulates hosts (in a broad sense, vectors, reservoirs, accidental hosts · · ·), physiology (with temperature), and behavior (with seasonality).

32.2 TOOLS AND STRATEGIES: AN INTEGRATIVE APPROACH

32.2.1 Choosing the Appropriate Strategies and Identifying Corresponding Tools

Choosing the appropriate strategies and identifying corresponding tools are the main paths in order to provide answers for complex situations of disease emergence. For the understanding and hopefully prevention of diseases emergence, field observation is essential. This makes it possible to examine the phenomena behind the process of emergence. The mechanisms may be biological, at molecular level, or behavioral at an individual or population level. However, observation of exemplary situations is not sufficient, to transform emergence into equations. There are too many parameters that intervene in the emergence of a disease. An epidemic is only one of the possible outcomes of a great number of possibilities of emergence and diffusion. The reduction of risk can only be considered in temporal and spatial terms which make the system stable, but which no longer corresponds to individuals. Only an approach integrating observation, simulation, and prevention will make it possible to locate these optimum domains in terms of time and space. Observation is necessary to describe and decipher the reality and understand the phenomena, simulation to evaluate possible danger and to find areas of stability, and prevention by modifying the parameters to decrease the vulnerability of population or the probability of a situation favorable to emergence.

32.2.1.1 Deterministic risk assessment An omnipresent misguided principle in research is that of the relation of cause and effect in nature. It is implicitly accepted when one seeks to understand causes of a phenomenon by highlighting the relations, factors, and behaviors, which explain a given situation. It should make it possible, in theory, to envisage the state of a system starting with initial conditions and the rules, which govern temporal evolution. The object of the majority of sciences is to determine these primary laws and conditions.

But there exists in nature considerable number of systems (in fact the majority, if not all), which do not satisfy the principle of determinism. Very often, a state cannot be deduced from initial conditions because the relation of cause to effect cannot be quantified with sufficient precision to ensure the determinism of the system. One would need, for that, an infinite precision for all parameters of the system. The majority of systems in nature such as the climate are dynamically unstable and their exact evolution is thus impossible to envisage with certainty.

32.2.1.2 Probabilistic risk assessment Even if the value of all parameters for participants entering the system is known, (position, displacement, etc.) its evolution cannot be extrapolated with certainty but only estimated in a probabilistic approach. A deterministic approach of individuals is impossible (the exact

behavior of a mosquito or a human cannot be predicted). Therefore, it is necessary to regroup agents (domains, social or environmental characteristics, temporal factors, etc.) or objects (subsystems, domains, etc.) in larger entities which evolution can be predicted with a greater probability. We thus seek to pass from a study of a dynamically unstable system to a more stable one by changing the study focus.

It is possible to give the probability of occurrence of an influenza epidemic in a given population but it is impossible to give the probability that a certain individual will be sick at a given time. The emergence and diffusion of diseases appears to have a similar mechanism as climate, but more complex, because the laws, behavior and initial conditions of agents are less known. On the contrary, it is easier to act on the initial conditions and the behaviors (by vaccinating the population, by giving rules of hygiene, by restricting displacements, etc.).

In the face of dangers represented by disease emergence, reemergence, or diffusion, the research and practice of a therapeutic solution are all the more effective if the disease development mechanisms in the population (human or animal) are known. Afterward the risk can be evaluated to avoid situations that are highly favorable to emergence or diffusion. Given the population, it is also necessary to describe their behavior, the various states induced by these behaviors, and the phenomena and significant parameters leading to a change of state.

Describing the system, questions arise

- Is it necessary to study individuals, or populations, and which ones? Which parameters should be taken into account?
- Is there sufficient scientific knowledge to study them?
- How can the agent's behavior and inter-relationships be described and interpreted?
- Finally, in reviewing the entire system/mechanism can a potential risk be removed?

32.2.2 The Emergence Play: Actors and Decors of a Drama

32.2.2.1 Agents and behaviors It is the risk (probability of an event) and not the actual event, which must be studied. A real medical situation is conceived only as a possible event among many others. The phenomena being studied have individuals as participants or dynamic agents causing evolution in the system. The agent's outcome is determined by its position; the interrelations and its condition, the whole is determined by rules of behavior (probabilistic). The change of states or the rules of behavior can be strongly influenced by the medium (which is not regarded as agent—not cause leading to effect—but which can also evolve over time. Thus, the climate is in general one of the principal factors in vector behavior).

32.2.2.2 From object to population It is the existence and unique characteristics of individual agents, their behavior and interrelations, which causes disease evolution in a population. But it is not possible to describe all individuals and to envisage all behaviors, even if one knew exactly the mechanisms of disease evolution. If one considers only populations, a statistical description would be satisfactory but behavioral variations between populations would be difficult if not impossible to describe and thus to use. In particular, when behavior depends on environmental conditions the system becomes too inaccurate to reflect real life. A deterministic approach, by discipline, cannot make an accurate study of disease emergence in populations. No system of equations will be able to answer the questions, because of the element of unpredictability at all levels. The principal difficulty consists in controlling as much as possible these random components. Biological and medical science intervenes in studying the pathogenic agents at individual level. Social sciences and geography intervene in studying agents' behavior; factors and invariants are found from regrouping agents and measuring environmental influence. The study of real situations enables us to identify and study the actors of the transmission (pathogens, vectors, hosts, humans), to make assumptions on rules (states and change of states) and behavior of actors (actions producing a change of state), to specify initial conditions, but they should not be perceived as more probable than other possible outcomes.

The rules of individual behavior, even if they are known to be uncertain, are necessary to take into account the total phenomenon: It is only on the scale of individuals that one will really be able to simulate the process to find the most significant invariants, without using arbitrary and often too simplistic regrouping.

32.2.2.3 Population or individual? It is difficult to avoid this contradiction among study scales; to preserve the study of individuals while avoiding any determinism on their level. This amounts to describing agents and their behavior in probability terms compared to actual values (in a statistical way on populations or descriptive on selected individuals). Of course, to assign to an individual values of the population to which it belongs (according to the probabilities determined by observation) cannot be valid other than within the framework of a model or simulation aiming at highlighting invariants (with, once again, the process ensuring the statistical validity of the results obtained).

32.2.3 Requiring and Acquiring Data: From Who, to Where and How?

The observation of a real situation is fundamental to try to define agents and rules of behavior, whether they are molecular or demographic. Biology, epidemiology, entomology, geography are all essential disciplines to decipher parameters in an epidemic situation.

Who? The study objects. It is necessary to describe several levels to understand phenomena, both at individual and population level. It is necessary to describe several systems, to describe their agents, their states and their behaviors, and to find relations or inferences which make it possible for the descriptive study to enrich knowledge in other fields. It is necessary to set up procedures of stochastic simulation

making it possible statistically to validate the inferences used between the various levels of descriptions.

Where? It can be useful to change the object of study, by privileging space and time rather than the individual or the population in the process of risk evaluation. The studied individual is then a spatial unit (or an aggregate of spatial units), and the objective is to evaluate the probability of this entity to be the place of disease emergence at a given time. But this is based on the supposition that at every moment the characteristics of this entity are known for its agents (pathogenic, human, vectors) and for all parameters intervening in the behavioral rules of these agents (natural environment, vicinity, domain, etc.). Here again the use of a stochastic model is necessary, because the source of information is not exhaustive and must be approached in probability terms.

How? In certain exemplary diseases this approach makes it possible to seek objectives and invariants, in terms of emergence and diffusion in time and space, which will make it possible to describe and hopefully avoid situations favorable to certain health situations. This systemic framework also makes it possible to focus on the problem of emergence, beyond such or such pathology (e.g., concept of rupture in balance of population's health stability and concept of pathocenosis).

What? Many tools are necessary for this approach: Tools of biological and medical science to evaluate the medical, biological, and behavioral characteristics of individuals. Tools related to the information sciences, to allow definition of the various agents, and then use them in a rational way. Mathematical and statistical tools, to evaluate certain agent's characteristics compared to the population. Tools for geography, to define and represent the real world and environment. Tools for space and temporal analysis, to manage the environment and evaluate certain agent characteristics compared to their environment. Tools for models and simulation, incorporating all characteristics of the studied system to detect temporal invariants. The geographical information systems perform an essential task of regrouping some of these functions.

32.2.4 Model and Simulation

A model is designed using information based on observation of a real or supposed situation. Each hypothesis will be represented by a unique model: It is formed by the definition of agents and their states, by rules of behavior (probabilistic) inducing changes of state, as well as variables resulting from random determination of states and behaviors.

Simulation makes it possible to implement the model to obtain "events" starting from initial conditions. These events are obtained in a nondeterministic way, by applying to each agent the appropriate behavior rules for each step of time. It makes it possible to describe the probability of clusters of events (in space and time), and to test the influence of change in certain parameters (public health, behavior) on these probabilities (rates of vaccinations, social behavior, etc.).

As it is impossible to deduce the state of a population from one individuals, and if one does not want to remain focused on the level of populations but to go to the finer scale of individuals, it is necessary to use a process of stochastic simulation, the state of each individual being evaluated in a random way according to its place within the population or of the place he comes from (to measure the influence of environmental factors). In such a process of simulation, the inference is statistically possible if it is repeated many times (to be statistically valid, according to a confidence interval) and if it stays within a framework of nondeterministic modeling. Thus, it will be possible to seek invariants evaluate the influence of certain factors (descriptive or behavioral) on the system evolution , and draw some results on the probability of disease emergence based on these factors.

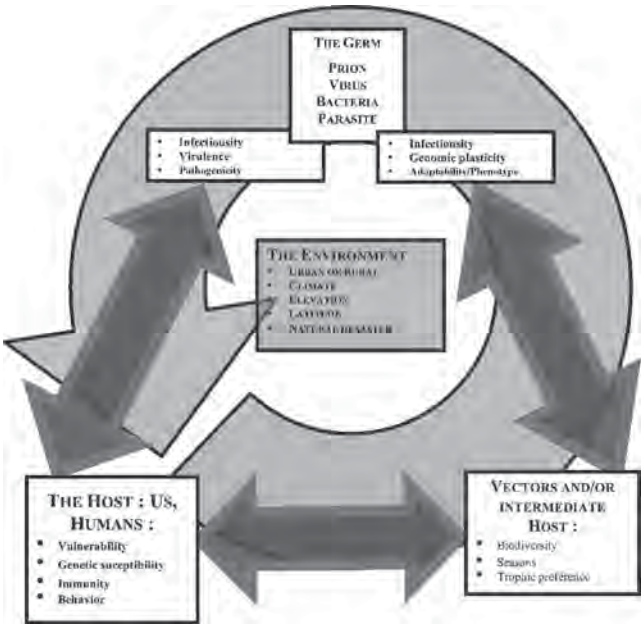

Fig. 32.2. The human infectious pathogenic system: a germ, a host, a vector, and an environment. The large blue arrow shows interactions: for example,: coevolution; selection (host immunity, germ lethality); behavior; migration (natural or forced). Hosts (infected by germs) can be accidental, intermediate, vectors, or reservoirs. Although some diseases are limited to a strict human cycle (human to human transmission , e.g., measles), the human host is often accidental in the context of disease emergence. The individual may belong to a population or sub population susceptible to infection and in a region or environment conducive to disease emergence. Blue arrows show interactions between "players." Green arrows represent environmental factors whether of natural or human origin. This model can also, with some modifications, apply to vertebrate animals (as in the emergence and reemergence of myxomatosis in the lagomorphs, the mad cow disease), and in invertebrate animals (e.g., the virus of leishmania—"parasite of the parasite"—in 1989), and even in plants (with the emergence of Geminivirus, discovered around 1990, transmitted by flies to tomato, cotton, and manioc plants).

32.3 EMERGENCE OF EXEMPLARY DISEASES OR SYSTEMS

Several diseases have been chosen because they show in a practical manner how the use of modern technologies applied to the study of emergence can produce an original

TABLE 32.2. Principle Indicators in Different Residential Areas for Some Developing Countries

Country	Environment	Infant Mortality (‰)	Children < 5-year-old Mortality (‰)	Immunization Coverage (%)[a]
Burkina Faso	Ouagadougou	69	119	68.4
	Rural	95	202	41.2
Cambodia (2000)	Urban	72.3	92.6	46.3
	Rural	95.7	126	39
Haïti (2000)	Metropolitan area	89.8	108.5	31.2
	Rural	90.5	149.4	33.5

[a] Children having received all vaccines: BCG (tuberculosis vaccine: bacillus Calmet & Guerin), measles, three doses of diphtheria–tetanus–whooping cough combine vaccine; poliomyelitis vaccine not included. However, this apparent advantage of the city over the countryside should be viewed with caution because cities being by nature heterogeneous are at the origin of significant intra urban health disparities [58].

understanding of its mechanisms and also help to develop strategies of prevention or prediction of diseases not yet emerged in a population and domain and for a period of given time.

The examples, which follow, were generally selected from work in progress at the time of drafting this chapter. In addition, it is shown that the cross-disciplinary approach is at the heart of analysis, to exceed the multidisciplinary element and juxtaposition of employed disciplines and to achieve a united thought process regardless of disciplines while emphasizing one or the other, a domain according to the angle chosen for the demonstration.

32.3.1 Assessing the Risk of Disease Emergence in a Changing World

Indicators observed in southern cities attest longer life expectancy; decreasing child mortality and better vaccine cover than in the countryside. Even if these indices pose problems of comparison, it is evident that urban situations create many differences depending to some extent on the presence of infrastructures and public services with greater accessibility. Better access to facilities like electricity, drinking water, education, and health care are also influential as are the possibilities of adequate housing and more job offers, and so on.

As a result of demographic and health surveys, ORC Macro.

32.3.1.1 Environment and the "Urban Case" The demographic transition resulted in an increase in life expectancy, which was accompanied by a marked change in the nature of medical problems in cities, particularly the larger ones. In northern countries, an evolution of cause of death has been observed; the first stage characterized by a prevalence of deaths from infectious or parasitic origin, was superseded by deaths from cancer, metabolic diseases, or cardiovascular infections. The simple model of "epidemiologic transition," imagined by Omran [51] no longer seems applicable to southern countries. Now South cities are also experiencing a real epidemiologic modification, characterized by the emergence of pathologies previously viewed as reserved for the North; like arterial hypertension, diabetes, the problems of mental health, traditional pathologies of incessant poverty, possibly in new forms.

Urban pathology, for the least developed countries, thus remains dominated by the infectious and parasitic problems, with first rank given to malaria, diarrhea, malnutrition, and respiratory infections. These traditional pathologies are in direct relation to problems of poverty and associated unhealthy living conditions.

For a long time it was thought that, urbanization would end the plague which malaria inflicted due to its incompatibility with the polluted urban environment and the ecological needs of anopheles, the mosquito-vector of malaria. However, it is recognized today that urban malaria exists [25]. Several studies show that it was actually transmitted in cities where the perimeters of irrigated cultures, imprint holes for the manufacture of bricks, the foot prints of cattle which frequent the banks of these water holes, all constitute the ideal breeding ground for anopheles.

Because of less dense vector population, transmission is weaker than in the countryside, but infection is often more severe and spread worse as the city dweller's immunity is much lower than that of villagers. In addition, transmission is unequal, depending on conditions varying from one district to another.

This is true not only for malaria but also for the schistosomiasis, which are transmitted in cities (The Dam at Aswan, Egypt) or for the African human trypanosomiasis (Sleeping sickness), which continues to prevail even in cities as was the case in the 1970's in Bamako or Conakry, in relation to crop areas (orchards in Bamako, cash crops in Conakry) and with proximity to rural surroundings [45].

32.3.1.1.1 Malaria in Ouagadougou (Fig. 32.3) Ouagadougou, capital of Burkina Faso, has approximately 1,200,000 inhabitants (more than 50% of total urban population). Malaria is responsible for 30 –40% of morbidity recorded in medical centers. A domiciliary investigation made recently in Ouagadougou shows that in children from 6 months to 12 years old, the average prevalence of malaria is 21.3% with great disparities in time and space [1]. In the irregular and sparsely built-up areas of Congo, prevalence exceeds 33.2% at the end of the rainy season whereas in regular densely built districts, it is recorded as less than 11% on average without increase in rainy season. There are however,

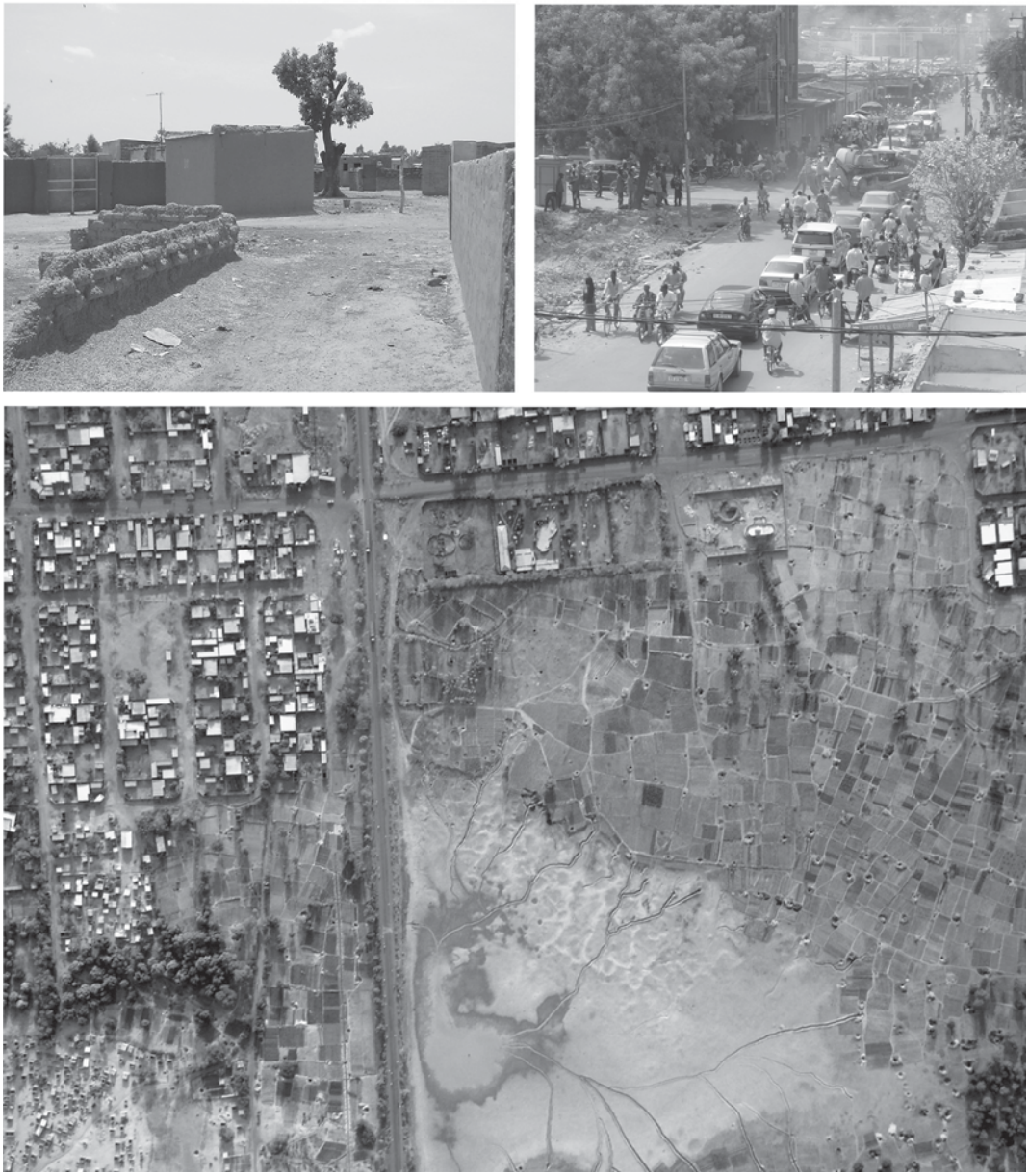

Fig. 32.3. Density of urban community in Ouagadougou and malarial risk. Photo C (below) at top, left, a regular district, which overlaps an irregular one (bottom, left). On the other side of the road (on the right), the dam Boulmiougou drained in April, fringed by small plots of crops for market gardening which generate a risk of malaria infection (habitats for anopheles) for the entire area's inhabitants (ONEA, 2003).

areas where risk levels are less distinct, populations of regular zones being subjected to similar risk as those of irregular zones. The regular less densely built districts like Tanghin and the irregular densely built districts represent intermediate environments with a prevalence rate of approximately 15%. The existence of significant numbers of anthropised larval habitats explains transmission of intra urban malaria. The banks of the dams provide places where anopheles find good conditions for survival, just as the orchards around the dams or the imprint holes for the manufacture of bricks as well as the wetted ground near to the numerous wells at Ouagadougou.

These cultivation methods are frequent, even in the large cities. In Accra, capital of Ghana, 90% of vegetables on sale are produced in the city. The presence of these crops not only on

the periphery but also in the city center contributes in breaking the decreasing gradient of malaria transmission of central districts to resemble the peripheries and allows transmission in almost any season depending on the area. If this prevalence is much weaker than in villages close to the capital (prevalence of more than 60%), it is noted that people adopted very different habits from the rural areas, on average one child in three sleeps under a mosquito net in the regular districts as in the poor districts and more than three children out of four had taken an anti malaria treatment 15 days preceding the survey. If traditional pathologies due to severe poverty persist in southern cities, possibly in new forms, as previously evoked with malaria at Ouagadougou, they now share the same diseases as northern cities, for example, diabetes and populations, particularly the most vulnerable, find themselves prey to a double burden of diseases.

These changes are explained by the evolution of styles of living, eating habits, people eat more meat, more sugar while reducing their energy expenditure. Social references are also upset. Along with these factors, a growing pollution of southern cities is added without proper provision for making health care a priority. The living conditions of new immigrants are difficult when recently arrived in the capital, it is necessary for them to find work, housing and be nourished. These new life styles generate a stress, which can be at the origin of pathologies related to the way of life like diabetes, certain cancers, or arterial hypertension.

32.3.1.2 Conditions of emergence, from one pathology to another: The respiratory infections in France During the twentieth century, France has experienced immense changes in the field of medicine. Considerable progress was achieved, which is reflected in the evolution of mortality. In one century, life expectancy increased by 30 years for men and 34 years for women. This strong progression was accompanied by a significant shift in the cause of death, and is seen in particular, in the evolution of respiratory mortality. Mortality by respiratory infections (all types of infections included) strongly decreased over this period. The rate was divided by three for men and 7.4 for women. This strong fall is primarily due to a collapse of mortality by infectious diseases of the breathing tract. The infectious component represented indeed more than 3/4 of mortality by respiratory diseases, in 1925, tuberculosis constituted a real plague killing nearly 90,000 people each year.

To the deplorable consequences of tuberculosis other quite as significant infections concerning the breathing apparatus are added. Fault of treatment available, at the beginning of the twentieth century pneumonia, influenza and the other acute affections of the respiratory tract appeared particularly fatal. The respiratory complications of affections considered as benign today were frightening and dreaded, affecting in particular, young children, old people, or those already weakened by chronic respiratory problems. The influenza epidemics caused significant fluctuations in death rates from one year to the other. It was not until the end of the 1970's, with the diffusion of a general-purpose vaccine, that the fluctuations from mortality by influenza start to disappear. The death rates by influenza are maintained today on a very low level.

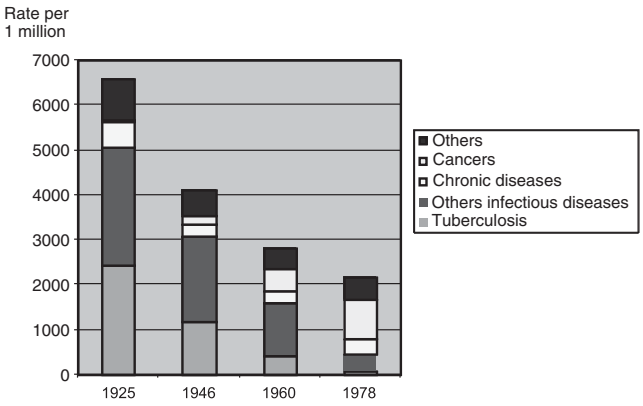

Fig. 32.4. Comparative rate evolution of male mortality by respiratory disease in France from 1925 to 197 (from Ref. [66]).

32.3.1.2.1 The growing burden of chronic affections and tumors The strong fall in respiratory infections is accompanied by a strong increase in mortality by cancer of the respiratory tract throughout the period and by an increase in the chronic affections at the end of this period (from the Sixties). Death rate by upper respiratory tract cancers was thus multiplied by 23 in 50 years. This rise primarily occurred between 1930 and 1970. Even if a share of this increase can be explained by the diagnostic progress, it testifies mainly to a considerable and regular development of the male nicotine addiction since the beginning of the century. The beginning of a decrease in nicotine consumption from 1975 resulted in a stagnation of levels since the Nineties. Minimal, even unimportant at the beginning of the century, cancers of the respiratory tract became today the first cause of male mortality by respiratory diseases. The development of female nicotinic habit, much more recent, causes a true increase in the death rates only as from the Eighties. These remain still today largely lower than the male rates. This late development saved France from a plague, which already affected the countries of northern Europe and the United States since a long time. After having touched the principal agglomerations, it extended today to all French cities. The expansion of female nicotine addiction, at least until the years 2000, makes inescapable the generalization of this phenomenon.

Risks associated with respiratory affections have shown deep upheavals during the last century. From a primarily infectious risk, French society passed in less than one century to behavior related risks. This change was accompanied by a reduction in respiratory affection mortality. Control of infectious risk was beginning to lead to the belief in the Sixties that respiratory problems were definitively solved. The diseases of respiratory tract were therefore put aside for a long time as medical priorities in France. Their development results from a late awakening as to the severe health consequences from the rise in nicotine addiction and industrialization.

In a preliminary conclusion, it is thus difficult to say that urban pubic health is better or worse than that of rural populations with so much intra urban disparities regional effects are significant. Concepts of epidemiologic and medical transition, which seek to give an account of this complex evolution, must also be revised because they are too normative and deterministic. It is necessary to allow for a period of reflection on general conditions of emergence and disappearance of diseases (in particular of coexistence and interactions, of exclusion) in any given environment. Geography is at the core of this initiative, by its capacity to aid in analysis of combinations of natural, social, economic, and cultural factors in a given place, to determine the different necessary levels of information and not to lose direction in a pseudo spatial epidemiology, which is unable to lend reason to its charts, or more basically to health inequalities.

32.3.2 Comprehension of Mechanisms of Emergence and Their Control

32.3.2.1. Defining proper spatial scales for dengue hemorrhagic fever According to a WHO (http//: www.whosea.org) annual report, infection with Dengue virus (DENV)—recorded for more than 100 countries—is the most widespread arboviral disease in the tropical zone, only restricted by deserts, oceans, cold, and elevated areas as its geographical borders.[6] DENV is mainly transmitted by *Aedes aegypti* a mosquito breeding in small artificial containers, such as jars, flower pots, cans, tires, frequent in urban environments and by *Aedes albopictus* in more rural areas.

The dengue virus belongs to the family of *Flaviviridae*, the same as Yellow Fever virus; it exists in four forms, (serotypes), and at least two circulate simultaneously in most endemic countries. An infection by one of the four serotypes induces in the host a specific and permanent immune protection for this serotype, no durable crossed protection toward the other serotypes, but a previously infected mother transfers a temporary protection (about 6 months) to her progeny. Dengue infections range from unapparent or nonspecific fever (DF) in more than 90% of cases, to severe forms such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). In Thailand, the mortality is lower than 0.2 %. DENV transmission exhibits a wide range of spatial and temporal variations related to the vector density, seasonal variations, differential level of herd immunity, and socio cultural parameters [35]. After several years, the spatial distribution of herd immunity in an endemic country resembles a patchwork where the propagation of each serotype will be more or less facilitated. Meanwhile, in Thailand cross-sectional serological surveys show that dengue infections mostly unapparent or undiagnosed reach more than 50% of the population (more than 90% of adults have experienced an infection by at least one serotype) and are incompletely reported; to be pertinent, epidemiological studies have to be based on records of DHF cases which account for less than 10% of the total infection but have characteristic symptoms and are generally hospitalized, and then recorded.

As neither treatment nor vaccine exists, the control of dengue fever is based on the reduction of vector populations, in particular by the community's efforts to eliminate potential breeding sites [71]. A permanent control of transmission over a whole country is not realistic, and a major goal for public health authorities is to identify periods and areas at risk. A strategy to control the disease should target risk factors such as the high-density vectors, areas of low herd immunity, and urban environments, which favor virus propagation. This approach can be taken at different epidemiological levels, from individuals to provinces, each providing specific types of information.

Since the 1990's, spatial studies have multiplied leading to the development of Geographical Information System (GIS), a powerful tool that stores spatial entities, and allows a wide range of analysis and included analysis in epidemiology [32]. Entities, such as houses are localized (georeferenced) thanks to their coordinates (latitude–longitude is the most common system) and characterized by attributes (e.g., house address; number of inhabitants; connection to water network; number of containers ⋯). In many countries, several geo-referenced databases exist with layers of information at different scales for the census, administrative limits, main roads, altitude, and type of land cover. DHF cases can be integrated in a GIS to generate incidence maps at different scales and time (e.g., seasons), or to describe the spread of an epidemic.

Daily records of hospitalization for dengue virus infection include information on patient address, date, age, and social status and on disease, severity, and treatment. The address leads to the place of habitation at the level of which one can observe clusters of cases, due to the short flying capacity of *A.aegypti* and the frequency of interrupted blood meals which allow a same vector to contaminate different family members. Moreover, the inhabitants, in proximity to their homes, create the majority of breeding sites for mosquitoes. At the community scale, the type of dwelling, connection to water network and water storage type are important factors for the creation of breeding sites, the number of which is estimated by classical entomological indices such as the Bréteau Index and House Index (respectively number of positive containers and number of houses with positive containers for 100 surveyed houses) used since decades and in many countries for dengue surveillance to classify villages and cities districts according to the density of positive containers. A major limitation is that larvae, on which these indices are based, face a high mortality during their development (because of competition for food, predators, drying up of containers) and different types of containers (e.g., a 0.2-L can or a 200-L jar) do not have the same probability to stay wet during the 5–6 days necessary to the full larval development; actually most of the positive breeding sites will not produce any vector. Larval indices appear then as not such an accurate way of estimating

[6]From *arthropod borne virus*: a group of viruses that needs an hematophagous arthropod vector (mosquitoes, ticks) to infect a new vertebrate host.

the number of vectors and the risk of transmission [8]. To improve the pertinence of entomological indices, WHO developed a multi country study to promote a surveillance system based on the identification of the types of breeding sites producing the majority of pupae. Pupae do not eat, have a low mortality before becoming adults and surveillance based on the containers producing pupae is therefore more likely than using those with larvae to inform on the number of potential vectors and the transmission risk in a given area.

As an example, information on each house collected during an exhaustive survey of breeding sites in a village (north–east of Thailand) were stored in a GIS, including GPS localization, number, type and productivity of containers. Results showed that 40% –50% of the positive container (with larvae) had also pupae and that the production by containers storing water for domestic use accounted for 90% of pupae. These containers were found regularly distributed over all houses, but during the dry season, houses located along the main street exhibited higher densities of breeding sites with pupae whereas peripheral houses distant of more than 110 m from any other houses were not colonized by *A. aegypti*.

Dwelling distribution; potential containers with pupae during the dry season in a village of north-east Thailand. (a) Dwellings distribution in a village of northeast Thailand. The intensity of the grey is proportional to the density of habitation (number of houses in a radius of 100 m around each house), varying from 1 to 25 houses. (b) Isolines represent potential density of breeding-sites (a number of potential larval sites in a radius of 100 m around each house), varying from 1 to 45 containers. (c) Isolines represent density of pupae (number of pupae in a radius of 100 m around each house), varying from 1 to 86 pupae. The black line marks the contour of the village at 100 m from the periphery dwellings.

The disappearance of the vector in isolated zones, in spite of the presence of potential breeding sites, is due to the stochastic pattern of the colonization by the females. The presence of water in containers is dependent on the activity of villagers and rainfall and is thus widely random. Each container can thus be emptied at any given moment, but with females laying their eggs one by one, in different containers, the production of adults can be maintained if the density of potential breeding sites is sufficient. In dry season the total number of water containers decreases and in isolated houses the females, whose flight is made more difficult by low air humidity, are brought to lay all their eggs in a small number of containers, whose drying out may lead to the local disappearance of the vector. At village scale the zones with higher dwelling density, share a greater number of potential breeding sites, which are unlikely to all, dry out simultaneously. According to Figure 32.1, the local disappearance of *A. aegypti* takes place when density of potential lodgings is lower than 3. 5 sites/ha.

However, transmission is probably homogenous in the whole village due to movements of humans and vectors in areas with different levels of risk. These observations could help to improve the control strategy: The exclusive treatment of the most productive containers in the most dense areas of villages could reduce the production of vectors with a better cost–efficacy ratio than the exhaustive treatment of all containers in every houses. Sociological patterns in relationship to environment must be taken into account to orient villagers in strategy for elimination of potential breeding sites, as most are filled by house owners.

Higher dwelling density also favor contact between vectors and hosts, whereas the presence of administrations, temples, markets, tourist areas, and proximity of main roads increase the risk of spread or importation of virus through travelers. Once a virus is introduced in a populations, infection involves the progressive immunization of potential hosts and the probability of an infested mosquito "finding" a susceptible host is likely to decrease until the transmission stops, whereas susceptible hosts are still present in the community, according to a similar stochastic process that causes interruption of pupae production in villages. Transmission in small communities is characterized by successions of epidemic periods due to emergence

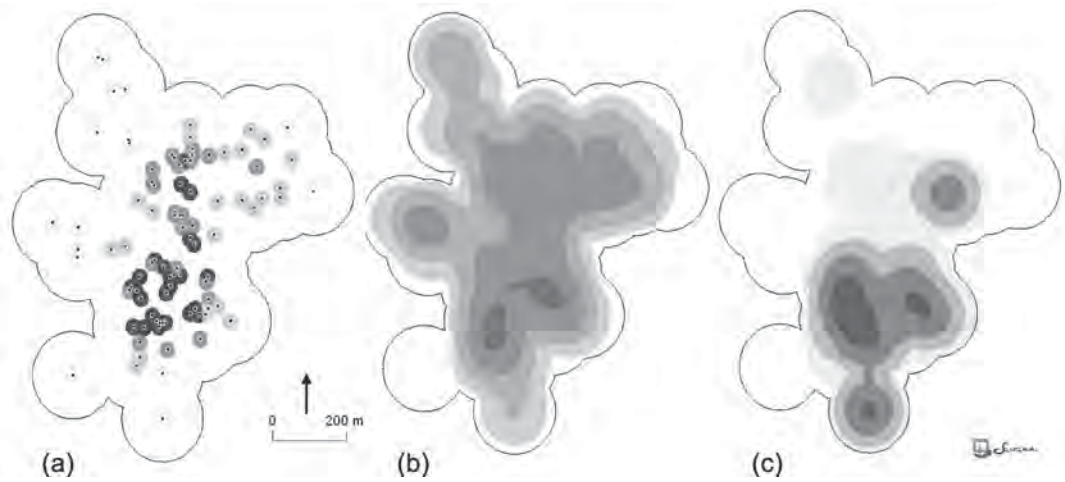

Fig. 32.5. Insights the dispersal heterogeneity of vectors seeking a breeding site.

of imported viruses, followed by long interruptions. Local interventions for elimination by ULV pesticides of females can thus be effective short-term, but must be carried out at the beginning of the epidemic, with the risk of being launched when the virus has already spontaneously disappeared. The development of control and prevention strategies implies then that incidence data are aggregated at a higher level. Cliff and Hagett [10] established empiric rules linking the size of host population (several tens of thousands of potential hosts are necessary to allow a sustainable endemic transmission) with waves of incidence or delay before interruption in transmission of infectious diseases and characteristics of the disease (measles, influenza). They also showed that the spread of a disease is a diffusion process dependant on the density of communities but also on distance between communities.

Information on density of human settlements and their distance from each other cannot easily be obtained from a census or map as the expected information is complex and may change rapidly: The actual limit of communities (that is not only an administrative definition or a dot on a map), number and density of dwellings in the different community sectors, distance from other communities, presence and size of roads. Another approach is based on satellite images to obtain an accurate description of the dwelling distribution. Rodgers and Randolph who delineated environments favorable for tsetse flies in Africa through information provided by the meta-analysis of the dense literature written on trypanosomiasis (sleeping sickness) during the twentieth century and the classification of vegetation provided by satellite images from Landsat and NOAAH did a precursor work using remote sensing data in epidemiology [6]. Such research opened a vast field of applications. In an attempt to adapt method to epidemiological studies in urban areas, a preliminary step is the use of satellite data for the estimation of distance and densities of places where potential cases live.

A classification performed on a Landsat image used geographical coordinates of several known inhabited areas to characterize the corresponding pixels[7] in the satellite image. A computer process using specific software is then initiated to find all pixels having same values of radiometry in the satellite image and enables the identification of all zones with (likely) same type of land use that is the inhabited areas. The result is confirmed by a comparison with the census giving a 90% correlation between the number of pixels and the number of inhabitants in each administrative entity (1 pixel = "*n*" inhabitants). For each subdistrict, we can calculate the population density by using the census associated to the satellite image (1 pixel = "*n*" inhabitants). Moreover, the distribution of "urban" pixels is not a random process but correspond to the expected urbanization structure: Beside some isolated pixels corresponding generally to farms or small hamlets, most are grouped, longitudinally (along roads) or in polygons (villages, cities).

[7]Pixel: the smallest spatial unit in a satellite image, where the land use is characterized by its radiometry measured by a satellite.

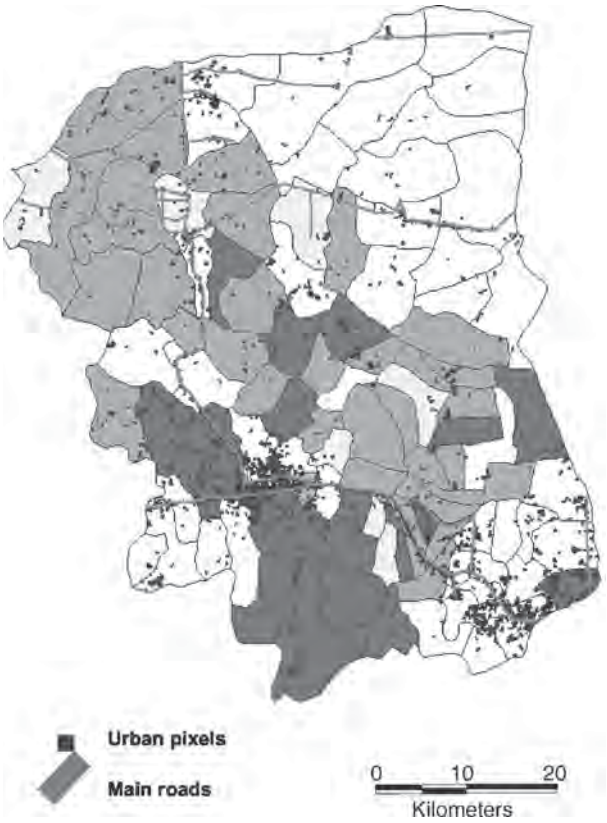

Fig. 32.6. "Urban pixels" distribution and the number of epidemic months among the 103 sub districts (1997–1998 Dengue outbreak, Nakhon Pathom Province, Thailand). Darker red corresponds to an increasing number of epidemic periods by sub district. Blue spots are aggregates or "urban pixels" obtained by the classification process of a Landsat satellite image. See color plates.

Figure 32.6, areas with medium density of urbanization (urban pixels) and distant from the main road, faced more epidemic events than areas with higher density of inhabitants. Development of herd immunity is a dynamic and heterogeneous process: Areas with outbreaks in 1997–1998, where relatively less affected during the precedent years, had a lower density of immunity, and therefore a higher risk to develop epidemics. After field investigations to quantify transmission (density of vectors, level of herd immunity) in specific communities, other communities presenting similar spatial characteristics can be identified on the satellite images (GIS) and epidemiologic results can (carefully) be extrapolated.

The study of spatial factors in the transmission of dengue thus highlights strong constraint, which structure transmission in large communities, mainly the density of vectors, the contagious process of transmission [2], and the density of susceptible hosts. However, in small communities, the low-flight capacity of vectors and social variability induce local heterogeneities. The females emerging in a given house will more frequently bite inhabitants of this house or nearby neighbors,

but can by chance, disappear. The severely sick individuals have to go to hospital where they are relatively isolated, but the healthy virus carriers will continue to move within their community contributing to locally spread the virus [49] but also to the constitution of a high density of immune hosts. Others can also traverse long distances thus taking part in spatial expansion of the virus. As a consequence of this discrete process, the contagious transmission pattern can reach any community, despite the vector's low-flight range (a few hundred meters for *A. aegypti*) and that most humans are globally sedentary.

Spatial analysis of dengue transmission using modern tools and technologies provides new insights on former studies and a better understanding of disease diffusion process. In the frame of control activities, the use of GIS and satellite images will allow identification of small communities with low probability of durable transmission where the community participation for the elimination of containers is traditionally well developed and that do not need to be preventively treated as they do not participate significantly to the virus diffusion. But they will largely benefit from the treatment of cities that will reduce the number of infective carriers potentially spreading the virus.

32.3.2.2 Japanese encephalitis: changing epidemiological pattern in a changing environment Japanese encephalitis (JE) is a mosquito transmitted viral disease (arbovirosis) due to the JE virus (JEV, *Flaviviridae* family). The basic selvatic cycle among birds involves ornithophilic mosquitoes and is in a large part responsible of the spatial extension of the disease during the twentieth century. The JEV is generally transmitted trough a domestic cycle to pigs by zoophilic mosquito species. Theses mosquitoes rarely bite humans whose infection is therefore accidental. Moreover, humans provide a parasitic impasse, as mosquitoes biting humans cannot be infected. Birds are resistant to the infection whereas infected pigs show high rate of abortion. The local virus transmission is closely associated with porcine farming, which has regional disparities (e.g. , size of farms, feeding pattern, structure of breeding); whereas pigs trade contributes to the disease spread.

In Thailand, after epidemics in northern provinces during the 1980's (several thousands of cases per year) the JE vaccination program was launched in 1990 and from 1997 has become part of the Expanded Program of Immunization in 28 provinces. The rare infections observed during the last decade (less than 100 per year) concerned mainly adults living or working in proximity of porcine farms, and where distributed over the whole country.

The context of transmission evolves concurrently with changes in agricultural practice. In particular huge industrial farms raising thousands of animals have replaced family farms with only a few dozen pigs. Theses farms being often located at short distance from inhabited communities create a risk of exposing nonimmunized population to the spread of the virus from areas of intense transmission among the pigs. An evaluation of this risk is necessary in order to develop preventive or reactive strategies.

The traditional cycle of transmission (during the major epidemics in the 60's and 70's) prevailing in Thailand was due to rural mosquitoes (mainly *Culex tritaeniorhynchus*, *C. gelidus*, *C. vishnui*, *C. fuscocephala*), developing in rice plantations or ponds, and biting mainly birds and domestic animals (buffalos, pigs). The population lived near cultivated areas, generally in hamlets or small villages gathering a few dozen traditional houses built on piles, each family keeping some domestic animals within its dwellings. However, sometimes the zoophilic mosquito attracted in the village by domestic animals could fed (about 2%) on human blood and thus transmit the JEV. Epidemics generally then break out quickly among the villagers living at short distance from each other, infected pigs being at the origin of virus persistence. However, virus disappearance was also rapid following the progressive immunization of pigs (human do not allow infection of mosquitoes), preventing the establishment of an endemic transmission. Virus first arrival in the villages was largely random, due to the pig trade at the origin of the introduction of infected pigs in villages (birds could also play a role).

During last decades, rural landscape has greatly changed. The small hamlets have progressively disappeared to the profit of larger villages, where inhabitants have easier access to services, schools, health centers, electricity, and water supplies . . . . In addition, agricultural practices have evolved toward greater intensification of farming. Considering factors in JEV transmission, villagers live at greater distance from rice plantations, family breeding of pigs has become rare, and transmission by rural vectors is therefore more difficult. Together with the vaccination, this has practically wiped out the disease in humans. On the contrary, the larger pig farms generally located in not densely inhabited areas for reasons of public health and safety are always in the flight path of *Culex* species from rice plantations. The transmissions among pigs is very intense and after 6 months of fattening in industrial farms nearly 100% of pigs arriving at the slaughterhouse are positive in JEV antibodies. Persistence of transmission is, again, made possible by the pig trade, but at the difference of the former situation, now the fast renewal of piglets in farms, which regularly bring new susceptible hosts, allows the durable maintenance of the virus circulation. Although pigs are more numerous, the number of humans in contact is much lower than it was when family breeding was practiced; they are the owner and farm employees, who now provide the majority of the rare JE cases since the last decade.

An alarming factor, however, is the presence in the industrial farms of *Culex quinquefasciatus*, the most widespread urban mosquito in tropical zone, and thus now in the large Thai villages, very anthropophilic for its blood feeding but also very opportunist, as this species also feeds on birds or pigs. Moreover, it is a confirmed vector of JEV, in the laboratory and in natural environment. The participation of this species to the transmission cycle of pig farms, associated to its presence in villages modifies the epidemiologic background and in the frame of constant increase in human settlement could cause the virus to be transmitted to large nonimmune, nonvaccinated human populations.

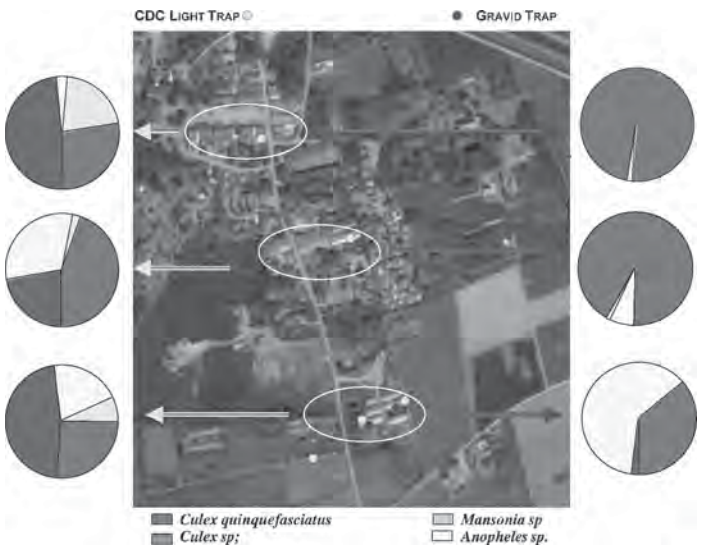

Fig. 32.7. Pig farm study site (obtained by Google° Earth). Two types of trap have been used: CDC Light trap (yellow dots) and CDC gravid trap (blue dots); white circle locates the pig farms which have been investigated. See color plates.

In view of this threat, various studies were undertaken to characterize and delineate risk and develop answers. One objective is to highlight variations in prevailing mosquito species according to environment, from rural to industrial farm to within villages and their risk to be in contact with unprotected populations. In an experiment developed in Thailand, mosquito traps were installed in pig farms then at increasing distances until the closest villages, generally located a few kilometers away. From analysis on engorged mosquitoes, the origin of the blood meal can be identified (ELISA techniques), whether from humans, pigs, or other vertebrate. Each trap site was georeferenced by GPS technology. Satellite high-resolution photography has been carried out (Google earth®) which allowed visualization of sites and their environment and evaluation of flight distances. Moreover, starting from this photography, various layers of information can be created and used through a SIG approach.

Species feeding only on pigs or that do not flight to inhabited areas are not very likely to transmit the JEV to humans, whereas exclusively anthropophilic species have little chance of being infested. The risk is in the diversity of behaviors. From these results, strategies for preventing and controlling the disease can be elaborated such as identification of the populations in need of vaccination or antimosquito activities. It may also be possible to make modifications in the pig trade. This example shows how, modification of environment, in this case of anthropic origin, modifies the sphere in which diseases emerge and develop.

In other situations, a change can be favorable, such as the draining of marshy areas in Southern Europe, during the twentieth century, which has contributed to the disappearance of local malaria transmission. However, special awareness must prevail as the increase in intensive agriculture together with rural migration implies dramatic modifications in environment and contact between populations (pathogens, hosts, vectors) that may have significant consequences on transmission of rural diseases. Another example is that because of intense use of insecticides for crop protection many species of mosquitoes, despite being not the target of interventions, have developed resistance to chemical insecticides.

32.3.3 Climate-Dependent Arboviroses

32.3.3.1 Dengue, aedes and climate: relationship between temperature and dengue haemorrhagic fever incidence Dynamics variations in dengue incidence range from low incidence endemic pattern to high incidence, the epidemic pattern and generally follow the seasonal variations, and longer cycles over 2–10 years.[8] Level of dengue virus transmission is a resultant of the dynamics of three interacting populations, host, virus, and vector [73]. Host dynamics beside demographic component (births, deaths, immigration) integrates the change from susceptible to infected and to immune status at a rate dependant on incidence. The virus is an obligatory intracellular organism, and its dynamics follow the number of infected hosts and vectors. The vector dynamics involve different phenomena: production of females in breeding sites, infection on infected hosts, and infection of susceptible hosts.

[8] cf C.2.1. for generalities on dengue

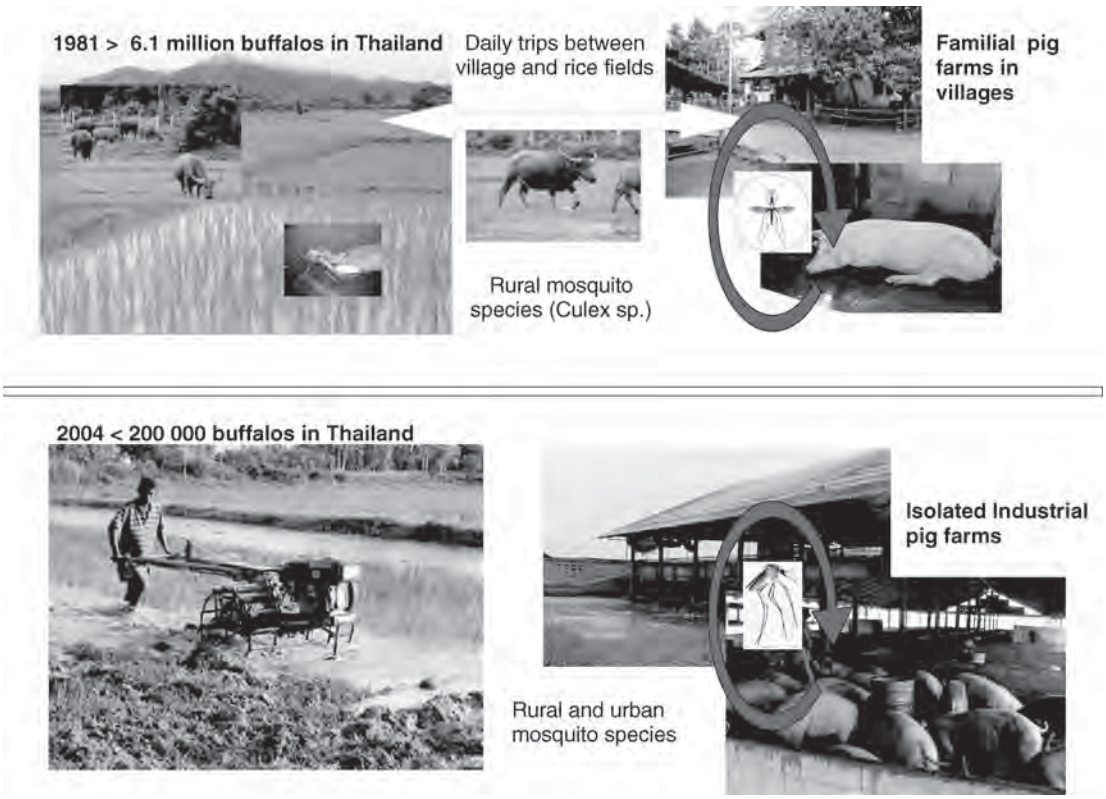

Fig. 32.8. Japanese encephalitis virus cycle of transmission in Thailand: past and present. The main JEV cycle in Thailand has evolved from a rural agricultural pattern to a village-pig raising farm environment.

The part of the cycle, which takes place in the vector, is subjected to weather variations with a direct impact on the density of vectors at several levels. The eggs of *A. aegypti* generally laid above water can wait several months for the first rains enabling their hatching as well as the creation of durable water filled breeding sites; a high temperature accelerates larval development, but increases evaporation in the breeding sites; high humidity help flight and supports survival and dispersion of adults. Moreover, high temperatures reduces the duration of the gonotrophique cycle (GC), period between two blood meals (for digestion of blood and maturation of eggs) and allows a greater number of blood meals during the life of a female; the duration of the extrinsic cycle of multiplication of the virus in the mosquito (extrinsic incubation period, EIP) is also reduced allowing for a greater number of females to become infectious.

The three principal climatic parameters, temperature, rain, and humidity are correlated but each one has proper dynamics: In tropical zone, the rainy season which follows the hot season is generally accompanied by a reduction in temperature; humidity decreases with high temperatures. Incidence also exhibits seasonal variations. In Thailand typically, the first rains lead to a rapid increase in dengue incidence, followed by a peak in the middle of the rainy season and then a slow decrease until a minimal value during the dry, cold season. The figure shows succession of these climatic variations and incidence variations observed in the north–east of Thailand. It is thus as a result of all climatic factors taken into account that the relation climate-transmission can be evaluated.

Complexity of those phenomena appears if we try to interpret the peak/decrease observed during the rainy season in endemic countries.

This pattern may be in large part driven by the variation in vectors number and probability to perform a complete EIP. The decrease in temperature affect different steps of the transmission potential (GC, EIP, and larval growth); heavy rains may wash up larvae and pupae from the small containers or decrease the density of food; populations of predators and pathogens of *A. aegypti* may stabilize at a high density level after a slow process due to the quasi-disappearance of *A. aegypti* during the dry season. But other factors are essential. Epidemics of infectious diseases (with no vector involved) generally follow a progression according to a bell-shaped curve: The number of cases increases in an explosive way, then reaches a threshold due to a progressive immunization of potential hosts and a low probability of the virus being transmitted to a susceptible host. The incidence then decreases quickly. The same phenomenon applies to arthropod-borne diseases and in the middle of the rainy season, after several months of intense transmission the density of susceptible hosts can have reached a threshold making further transmission difficult.

Several factors vary simultaneously and the role of temperature alone is difficult to quantify. However, constants, thresholds, and tendencies can be described.

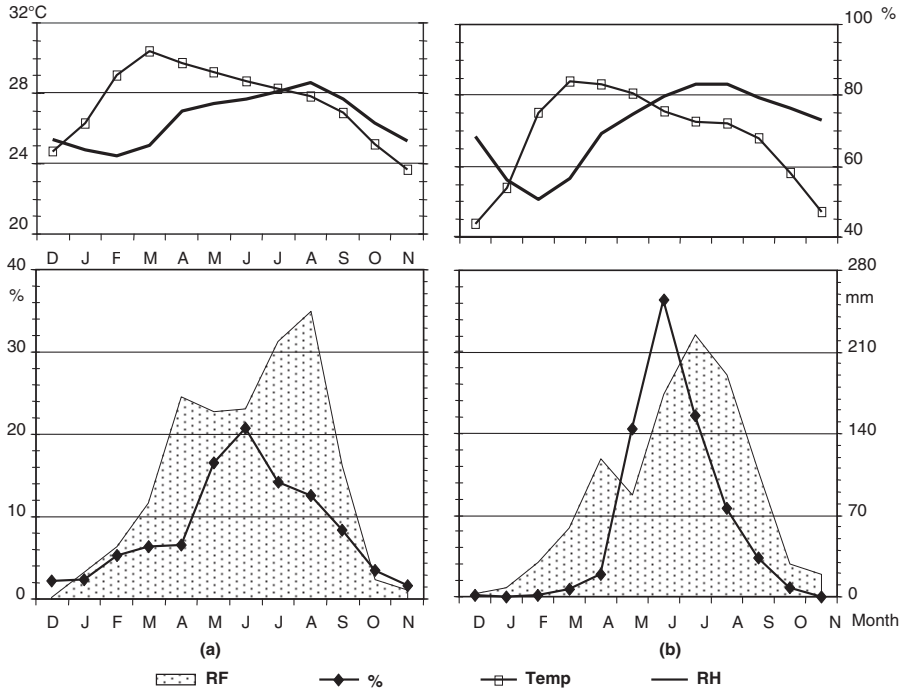

Fig. 32.9. Seasonality of DHF transmission in two provinces of Thailand according to climatic factors. (a) North–east province of Thailand (seasonality little marked, winter temperature higher than the transmission threshold); (b) Northern province (seasonality marked by a winter temperature located below transmission threshold < 22°C).

- Laboratory analysis showed that an increase of 10°C, within the limits compatible with vector survival, that is between 20° and 35°C, approximately divides in half the duration of the phenomenon considered: GC, EIP, and larval development.
- Each year the seasonal drop in number of cases intervenes, during the rainy season, when the temperature reaches between 27° and 28.5°C, whatever the accumulated incidence since the beginning of the period of transmission, and thus independently of the level of immunity reached by the population.
- In districts of northern Thailand located at an altitude higher than 600 m (Fig. 32.9b), the transmission is stopped during 4–6 months of the cold, dry season during which the rain decreases then stops. The monthly average temperature drops until falling below 21°C for 2–3 months, then rises until the first rains in May. An essential difference with the situation observed in Figure 32.9a is that virus circulation is stopped for this long cold period. As a consequence, the resumption of the transmission is delayed for several weeks after the first rains, corresponding on the one hand to the reconstitution of vector population but also for reemergence of the virus, initiated by viruses imported by travelers or transmitted by vectors resulting from eggs infected by females of the preceding generation.

It has been observed however, since the last years that the duration of this period without transmission has been significantly reduced despite the total yearly incidence being lower. On the contrary, the generalization of the water supply allows for a greater number of breeding sites to remain productive during the dry season, but it is especially the appreciable increase in minimal temperatures observed during the cold season, which makes it possible to avoid the disappearance of the virus. This illustration of a possible effect of global warming, must however be considered in the context of a country of endemic transmission of the dengue viruses. A high temperature would not be enough to allow virus transmission of the dengue in any area, even tropical: the vector must be present, and the social and cultural context, in particular the habitat, must allow a close contact between vectors and hosts.

The global warming is not the only factor likely to change the epidemiological situation, the enlargement of the area colonized by *A. albopictus,* is also highly challenging health authorities. The Asian tropical rural vector of dengue virus is becoming more urban, breeding in tires, which trade has largely contributed to its dispersal and over the last decennia has spread to Europe, USA, Southern America, and Africa. In these areas, the low level of herd immunity could favor the propagation of dramatic outbreaks.

32.3.3.2 The Rift Valley Fever: A climate – related arbovirosis in senegal Zoonosis with vectorial transmission are all, more or less, dependent on the climate which can be defined or characterized by all the conditions combined such as the air or water temperatures (maximum, minimal, average), the winds (intensity, direction), the relative humidity and its daily and seasonal variations, sun, rain (intensity, mode, a number of

rainy days, total quantity and distribution), and others. These conditions have, significant role in epidemiologic cycles, largely due to the vector biology. Among arbovirosis, those, which are transmitted by mosquitoes, having aquatic larval stages, represent a category of particularly climate-dependent diseases. In recent years, some have earned considerable importance: emergent in North America (West Nile virus), reemergent in West Africa (Yellow Fever), or in Southern Asia (dengue); or expanding in many countries of Africa and recently in the Arabic Peninsula (Rift Valley Fever, originating in East Africa).

To analyze relationships between environmental conditions and evolution of these diseases, we studied the fever of the Rift Valley in central Senegal, where it emerged in epidemic form in 1987. Several species of mosquitoes were found carrying the virus but two of them can be regarded as the endemic and/or epidemic vectors: *Aedes vexans arabiensis* and *Culex poicilipes*. Ferlo, a Sahelian area of approximately 60,000 km^2, represents a zone of extreme importance for the herds of ruminants (bovine, ovine and caprine), which can traverse hundreds of kilometres in search of fertile pastures. The appearance of these pastures is related to rains which, also fill the many temporary ponds of the area, provide water for animals, and stimulate development of sometimes extremely abundant populations of mosquitoes, able to transmit many viruses including the Rift Valley Fever to both animals and humans.

32.3.3.2.1 Climatic environment and ciral transmission risk Our studies relate to the ecology of these two species (73 % of the mosquitoes present) in relation to rainfall: How the rains can modify conditions of virus transmission of a mosquito to a host population? In this area of the Sahel the rain season is short (4 months), and variable from one year to another, (e.g., 2002 annual rainfall reached 299.1 mm; and 379 mm in 2003, showing a deficit of respectively 25 to 5 %

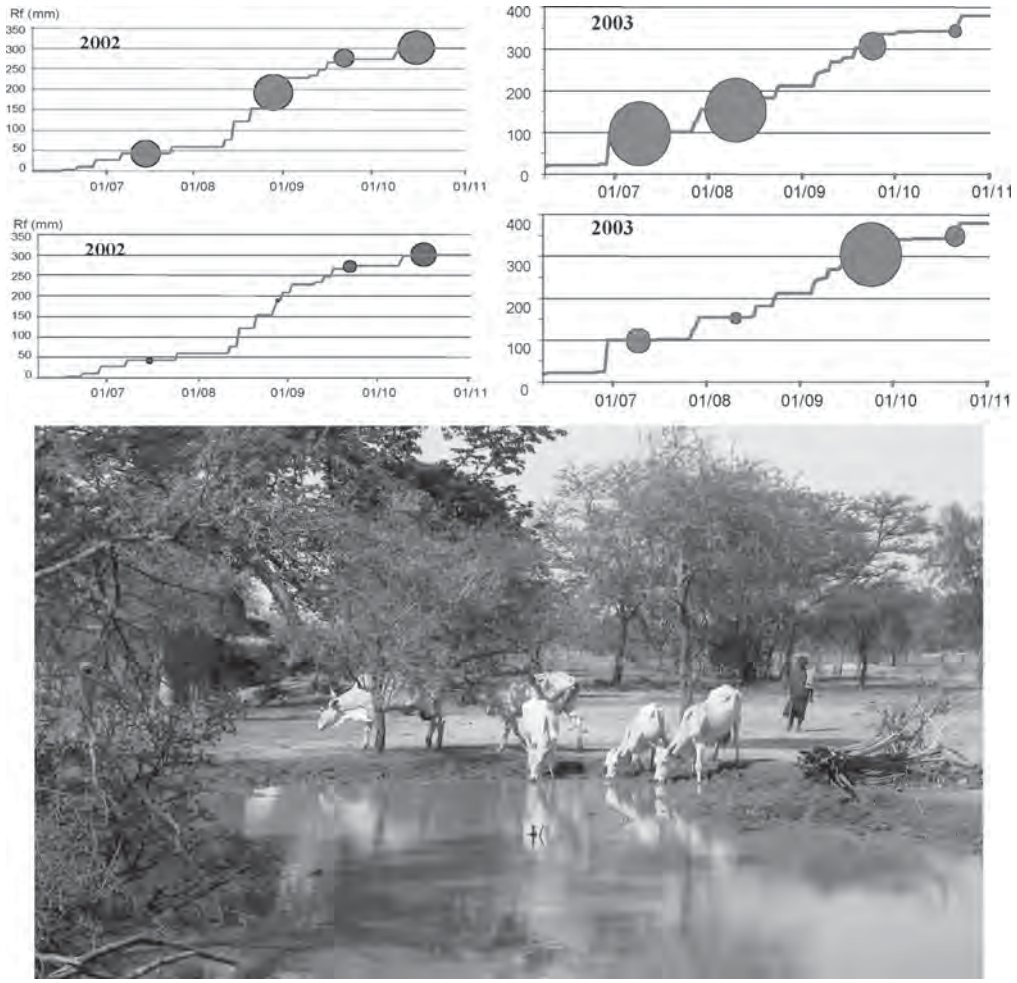

Fig. 32.10. Breeding site dynamics of Rift Valley Fever vectors: evolution of cumulative rainfall and abundance variations of *A. v. arabiensis* and *C. poicilipes* females. During the rainy season of the Sahelian region of Barkedji (Ferlo, Senegal) in 2002 and 2003, mosquito-breeding sites have been explored for four periods of 10 days. Red line , Cumulative rainfall; Green circle , Relative quantities of *A. v. arabiensis* females; Brown circle , Relative quantities of *Culex poicilipe* females; Relative numbers of *C. poicilipes* females; Relative numbers of *A. v. arabiensis* females; Cumulative rainfall. The surface water used by cattle are mainly temporary ponds where Rift Valley Fever Virus Vectors are breeding during rainy season in the province (Ferlo, Senegal). See color plates.

from the average over the last 40 years). By studying, in parallel, the variations of adult female populations and the characteristics of the rainy season, we tried to connect these data between them and to consider the extent to which a rainy season characterized by a certain quantity of cumulated water, a number of days of rain, their duration, rate or rhythm could stimulate, or not, an endemic or epidemic transmission of the Rift Valley virus to domestic animals and humans.

32.3.3.2.2 The survival adaptations of mosquito vectors

After emergence, females, once fertilized, take a first blood meal essential to the formation of eggs which will be laid at water level of the temporary ponds, either on wet ground close to water/air interface (case of *Aedes vexans*) or on the water surface (case of *Culex* sp.). If the eggs of *Aedes* are not immersed in water (no or very weak rains), they can wait several weeks even several months, until the rains of the following year to hatch, spending the period of dryness in quiescence. On the contrary, the great majority of eggs of *Aedes*, will not be able to hatch before having undergone a certain drying period, estimated at one week approximately, which corresponds to the duration of their embryogenesis.

The eggs of *C. poicilipes* laid in a raft by the female on the surface of water cannot enter in quiescence: They hatch on finishing embryogenesis or die if the pond has previously dried out. However, the appearance of a few adult female *Culex*, shortly after first rains of the wet season, after a period of dryness lasting sometimes up to 8 months, attests to the capacity of some females to resist the dry season in shelters of particular microclimatic conditions. The adults of *Ae. vexans*, appear only the fourth or fifth day after the first rains filling the ponds. *Culex* and *Aedes* thus have two different methods to resist the adverse conditions of the dry season: In the first case by surviving through some resistant adults and in the second case surviving from the egg populations, which can be extremely dense and which are ready to hatch with the first rain. The dynamics of these two populations will therefore be quite different.

32.3.3.2.3 Rainfall and vector population dynamic

Comparison of rain diagrams with relative numbers of *Cx. poicilipes* and *Ae. vexans* females during rainy season of 2002 and 2003 will enable us to understand dynamic these two vector populations.

At the beginning of a rainy season, the number of *Culex* females is low and they require stable ponds with sufficient water level so that the species biological cycle can be established: The first females can each lay a hundred eggs in one cycle and complete a cycle in 10 days, but the establishment of stable *Culex* populations may take several weeks. On the contrary, at the beginning of the rainy season, *Aedes* are potentially extremely abundant in the form of quiescent eggs. The quantity of females will depend on the surface area of ponds flooded after the first rains, as eggs laid by the females the previous year are distributed all over the pond surface.

Evolution of *Cx. poicilipes* populations is thus related to a constant filling of the breeding sites, whereas the dynamics of *Ae. vexans*, more complex, will depend on the quantities of rainwater but also on the period separating two rains, which must exceed the duration of embryogenesis to permit the eggs hatching.

Observations made during these studies can enable us to associate a type of rainy season to the potential risk of viral transmission. The actual beginning of the rainy season seems particularly significant: If the rains are abundant from the start (as in 2003), most eggs may hatch simultaneously and the population of *Ae. vexans* will quickly be very abundant, the risks of emergence of the virus even if it is still rare will be very high. In addition, significant rainfall means that pastures develop quickly, thus encouraging the early arrival of many herds of transhumant ruminants. If, on the contrary (year 2002), the rains at the beginning of the season are weak and scattered, *Aedes* populations will remain on a low level and the risks of viral emergence will be much less.

During the next weeks, the quantity of *Aedes* female will depend on the number of eggs laid by the first females of the year; so for those which hatch, a lag between rains of at least a week is necessary, which is seldom the case. The eggs, which do not hatch, will provide stocks able to wait until next year's rainy season. Then, the evaporation of the water of the ponds being generally higher than the rainwater input, no more egg hatching is possible. But, if as in 2002 (and a little less in 2003), significant late rains occur, the level of the ponds rise again, and a new batch of *Ae. vexans* will appear at the end of the rainy season. Herds being numerous, the risk of virus transmission from viremic animals to nonimmune animals increases and this is the traditional period of epidemics. Moreover, the risk of vertical transmission from an infected mosquito female *Aedes* to her progeny also increases, the virus can be maintained in eggs and re emerges quickly from *Aedes* the following year as soon as the rainy season begins.

The role of *C. poicilipes* appears simpler and in particular restrained to the transmission in the second half of the rainy season, the only period during which this mosquito species is abundant. The two species play then different epidemiologic roles. *Ae. vexans* has one of initiating the epidemiologic cycle and virus amplification at the beginning of year, then virus circulation at the end of the year and its persistence in environment through vertical transmission from one year to another; *C. poicilipes* contributes to the circulation and the risk of outbreak at the middle and end of the rainy season.

It can be assumed therefore that, provided that the serologic state of sedentary and transhumant herds of ruminants with respect to the virus of RVF is known, and that the rainfall records are quickly made available, it is possible to predict the risks in a rather precise way, according to the likely abundance and dynamics of the two main vector species populations.

32.3.4 Rain, Rodent, and Rice: Leptospirosis Epidemics in Thailand

After 8 years of epidemics, affecting thousands of people yearly with the highest incidence in 2000 (14,285 cases), leptospirosis seems to be fading in the epidemiological records to the

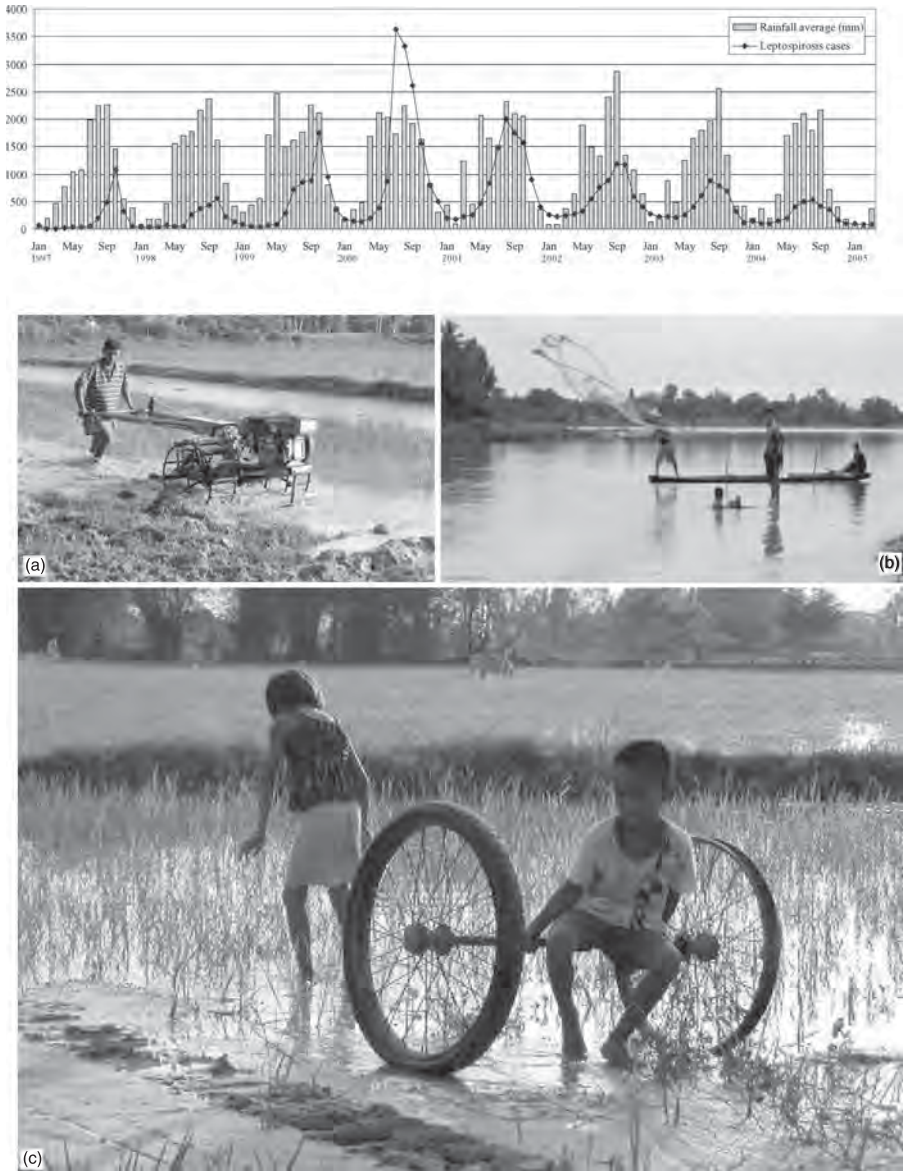

Fig. 32.11. Leptospirosis and water in Thailand. *Top Graphic*: Monthly rainfall and reported cases of leptospirosis in Thailand (January 1997–March 2005. (Source: Thai Ministry of Public Health, Thai Meteorological Department.) Three types of potential exposure to leptospirosis infection: (A) plowing in flooded rice fields at Udon Thani; (B) fishing at Kalasin; (C) recreation in rice fields.

satisfaction of public health officers and local communities. With a total of 1202 deaths from 1997 to 2004 mainly in the Northeast, Northern, and Southern regions, leptospirosis had become a major threat in rural villages. As an occupational disease, it has showed a specific vulnerability, as 75% of the cases are men and farmers. Prevention has been the main strategy of the Ministry of Public Health (MOPH) and its provincial and local offices to control the epidemics of leptospirosis.

32.3.4.1 Rapid emergence and decline of leptospirosis: marked patterns in space and time Leptospirosis is a worldwide zoonosis, occurring mostly in rodents from tropical and subtropical countries, where high rainfall helps the transmission of the pathogenic bacteria, from animals to humans, directly, or through water contaminated by rodent's urine. In Thailand, leptospirosis has shown a pattern typical of infectious disease epidemics, a rapid emergence since 1997 and decline starting in 2001. Leptospirosis is a seasonal disease, amplified in incidence during and after the rainy season, from June to October and later in the southern region.

In collaboration with the Ministry of Public Health , an active mapping was set up to produce monthly maps of leptospirosis incidence as a surveillance and control tool. Monthly cases, recorded by the MOPH from January 2000, were aggregated by month and incidences (for 100,000 population) calculated at district level, using population data

from the National Statistical Office. Monthly rainfall, recorded by the Thai Meteorological Department in 1250 stations, is spatially interpolated over the country to calculate the average by district. All data are regularly integrated into a Geographic Information System (GIS, *SavGIS*®).[9] For the whole country, a positive correlation (0.49; $p < 0.0001$) between rainfall and leptospirosis incidences illustrates the action of long rainfall periods, amplifying the range of infections, by spreading the bacteria and exposing rural population to higher risk.

Spatial and dynamic epidemiology show marked patterns with the highest incidences recorded in the north-eastern and northern regions with a near one month gap between the monsoon rainfall and the recorded cases. Some sporadic spots of high incidences are recorded later in the year in the southern region, also in relation to the longer rainy season.

32.3.4.2 From observed to real incidences: difficulties in assessing the extent of an epidemic Although retrospectively studying a disease, based on either epidemiological records or researches and laboratory investigations of human or animal prevalence, recurrent difficulties lie in comparing results obtained with evolving techniques. Back before the emergence of leptospirosis, serological investigations in humans or rodents have fed research work on the identification of a growing number of *Leptospira* serotypes. For instance, immunofluorescent techniques used in histopathology for the visualization of *Leptospira* in kidney sections are specific of serotypes, expected according to the rodent species involved and the ecosystem where it was trapped. However, the spread of an unexpected serotype would lead to false negative diagnosis.

Epidemiological records show main biases related to the health system, structure and quality, to the frequentation of health services by local populations and also to the difficult diagnosis of Leptospirosis presenting symptoms similar to fevers with other etiologies, such as dengue fever or scrub typhus. Before the first large epidemics in 1997, the clinical diagnosis was not sufficiently specific and the number of cases was possibly underestimated. Screening tests on suspected patients were generalized in 2001, each provincial hospital sending blood samples to a regional laboratory. It explains in part the decrease in incidence as confirmed cases were recorded the same year, while in 2000 the high concern for leptospirosis epidemics and awareness of the health sector contributed to attribute unknown fevers to leptospirosis cases.

32.3.4.3. Investigating hazard and exposure to understand leptospirosis dynamics Higher density and diversity in species prevalence of rodents explains the higher incidences in the north-eastern region compared to very low incidences in the central plain, despite intensive rice culture. Serological investigations of leptospirosis in rodents have shown positive cases throughout the country and have never distinguished spatial patterns of prevalence [7, 33, 65]. Several rodent species, although occurring throughout the country, were found positive: *Bandicota indica*, *B. savilei*, *Rattus rattus*, *R. exulans*, *R. norvegicus*, *R. argentiventer*, and *R. losea*. Other species were never tested in sufficient number to make any assumption relating to their noninvolvement in the transmission of leptospirosis. Not only were different serotypes of *Leptospira interrogans* identified in rodents, some relatively common like *L. javanica*, *L. autumnalis*, *L. bataviae*, and *L. pyrogenes*, but some other serotypes were also identified such as *L. canicola*, common in dogs, and *L. Pomona*, common in pigs and cattle, showing the proximity with domestic animals and the dynamics of bacteria, possibly infecting different vectors [7, 26, Herbreteau et al., 2005, unpublished data]. Therefore, the pathogenic bacteria present a low-host specificity, increasing the range of colonized habitats. Rodents inhabit most of the biotopes in Thailand, from natural biotopes, fields, gardens to houses. In the cycle of transmission, rain and standing water are a critical factor of transmission, maintaining the bacteria and also spreading them in contact with humans. Wet areas, flooded fields, and especially rice fields, are probably the main places of transmission. In order to delimit the leptospirosis risk, related to the rodent presence, tools have been developed using *SavGIS*® and its integrated remote Sensing module (RS) [26]. The basic principle of such process is to delimit the potential habitats of the main vectors of leptospirosis, which represent also the hazard in the risk assessment. The land cover is analyzed and interpreted as land use from high spatial resolution satellite images, using different vegetation indices. Such maps of the potential distribution of rodent species can help to target surveillance in risk areas and plan preventive actions.

32.3.4.4. Decline of leptospirosis: the role of prevention and public awareness Immunity is usually a major factor explaining the decline of emerging diseases. In the case of leptospirosis, it seems that serotype-specific antibodies are protective and that a patient is immune to reinfection with the same serotype. On the contrary, over 200 pathogenic serotype divided into 25 serogroups have been described. This huge diversity has also been found during serological surveys, where different serotypes were generally identified in the same areas, either in rodent vectors or in patients. So, immunity may only have played a minor role in leptospirosis decline.

Since the first epidemics, the MOPH has conducted active prevention campaigns to increase public awareness of the situations of high exposure to leptospirosis infection. They have recommended to the farmers to wear protective equipment, such as boots and gloves, as well as teaching the younger inhabitants of the danger of walking barefooted in any puddle or wet area.

Teachers taught songs about the "Rat's urine disease" (in Thai: "rok chee nu"), and villagers were informed during plenary meetings in the courtyard of dispensaries. Thanks to

[9] *SavGIS*® is a Geographic Information System (GIS) freeware developed by the French Development Research Institute (IRD, Marc Souris).

these efforts, leptospirosis is no more a mysterious disease for any Thai citizen. Even if working with boots in paddy can be very tricky, exposure has been reduced.

At last, easier access to health care has increased in treating fevers, allowing a better and faster treatment. If leptospirosis remains an environmental threat for farmers, a better surveillance in focused endemic areas and improved health care have contributed to its exemplary decline, at least in the epidemiological records.

32.3.5 New Pathogens, New Diseases: A Faunistic Approach to Reservoirs and Their Hosts

Ticks are also vectors of infectious diseases and participate in the emergence of several viruses belonging mainly to the rickettsia families (since 1991 more than 12 tick-borne rickettsial infections have been discovered.). Also, ticks can transmit bacteria and protozoa.

The "Lyme disease," a seasonal polyarthritis of New England, appears as one of the most emblematic of emerging tick-borne viral diseases from the past decade: The disease appeared in the eponymic county of Connecticut state and was associated to Ixodes tick genus and wild ungulates. *Ixodes scapularis* and *Ix. pacificus* are recognized as the vectors[10] and the Virginian deer (*Odocoileus virginatus*) as a main host for adult ticks whereas rodents (*Tamia stratus* or *Peropmiscus leucopus*), and, when rodents are in low density, birds (*Turdus migratorius*), or domestic dogs can be vicarious (secondary) hosts.

To date among the 869 tick species known from around the World, 53 species have been identified in Thailand, and are present in the Oriental biogeographical region. The particularly marked biodiversity known in Southeast Asia is also expressed in its number of *Acaridae* species.

Our objective in Thailand was, to produce an inventory of tick species with reference to those of medical importance. Most of Thailand has been explored and specimen collected, new species and new distribution described. Altogether using the entire literature available on the subject and our georeferenced records, a precise tick species distribution was provided. In order to obtain such degree of precision, beside a precise taxonomy, place of collection, date, and hosts were recorded. Using a Geographical Information System (GIS Savane freeware), we were able to generate maps, which will ultimately be used for risk evaluation of tick-borne transmitted diseases in Thailand.

The comprehensive approach of tick-borne diseases transmission requires knowledge of tick ecology as well as their vertebrate hosts and their seasonal geographic distribution. Even if the host–ectoparasite specificity appears determinant, it is known that for a region one virus can be transmitted not only by one tick species but also by another tick species, in another region, mostly when climatic factors are different, and that another vertebrate host will replace the former from a different biogeographical domain.

Such dynamic epidemiology will also need to take human behavior into account in order to evaluate the risk of tick-borne disease transmission to *in fine* propose prevention and control strategies. The table below presents the biodiversity of ticks and hosts regarding the arthropod-borne viruses transmitted by ticks and belonging to the biogeographical domain of south Asia.

The Langat virus strategies: Using the most abundant vector in the appropriate territory: The Langat virus (flavivirus) is responsible for meningo-encephalitis in humans, with a rodent reservoir of virus in Thailand, Malaysia, and Siberia. In nature, *Ixodes spp*. are the vectors from one vertebrate to another: *Ixodes granulatus* in Malaysia, *Ixodes persulcatus* in Siberia, and *Haemaphysalis papuana* in Thailand are the main vectors of the Langat virus. (I) Underlined are tick species present in Thailand; (II) probably surviving after accidental infection, potential to transmit; (III) spp (number of unidentified species).

Finally, if several territories like Europe or North America have been well documented regarding acarina fauna and their associated diseases, tick inventory and spread are very scarce for Asia at large, where studies have been limited and/or data not published. The present inventory and distribution are unique and pioneer the domain for Thailand. [64]. It is the first comprehensive biogeography of ticks from Thailand including potential vectors of human and animal diseases in Thailand.

The number of arboviruses isolated in Asia can be estimated at 126 (53 are transmitted by ticks and 30 of them are not known as pathogenic for vertebrates) among a total of 526 known to date for the world. Most of them belong to the *Flaviridae* and *Bunyaviridae* families. Finally, 15 tick species can transmit a virus to humans in ASIA and 14 of them are present in Thailand. The present maps show a georeferenced distribution of ticks by species and can be compared to the table for preliminary risk assessment.

32.3.5.1 The filovirus: a cirus family without host or reservoir—first evidence on ebola virus natural reservoir

Since the appearance of the first epidemic in 1976 and the first description of Ebola virus, many studies were undertaken in order to solve the mystery of the natural history of Ebola virus in Africa and to understand in priority, the emergence of epidemics so devastating to man and then also in nonhuman primates [14,46]. The natural history can be defined by viewing the whole system of transmission methods and virus circulation in its natural environment from its natural host (or reservoir) to humans. It thus includes determination of the natural Ebola virus reservoir, circulation of the virus within the population of its natural host, the passage of the virus to intermediate animal species, sensitive to the virus (i.e., developing a lethal infection), and finally the contamination of humans from the natural host or from the intermediate species. This text gives a progress report on all of these stages.

[10] In Europe and Asia, others vectors transmit Lyme disease respectively, *Ix. ricinus*, and *Ix. persulcatus*, which spread will map a risk geography.

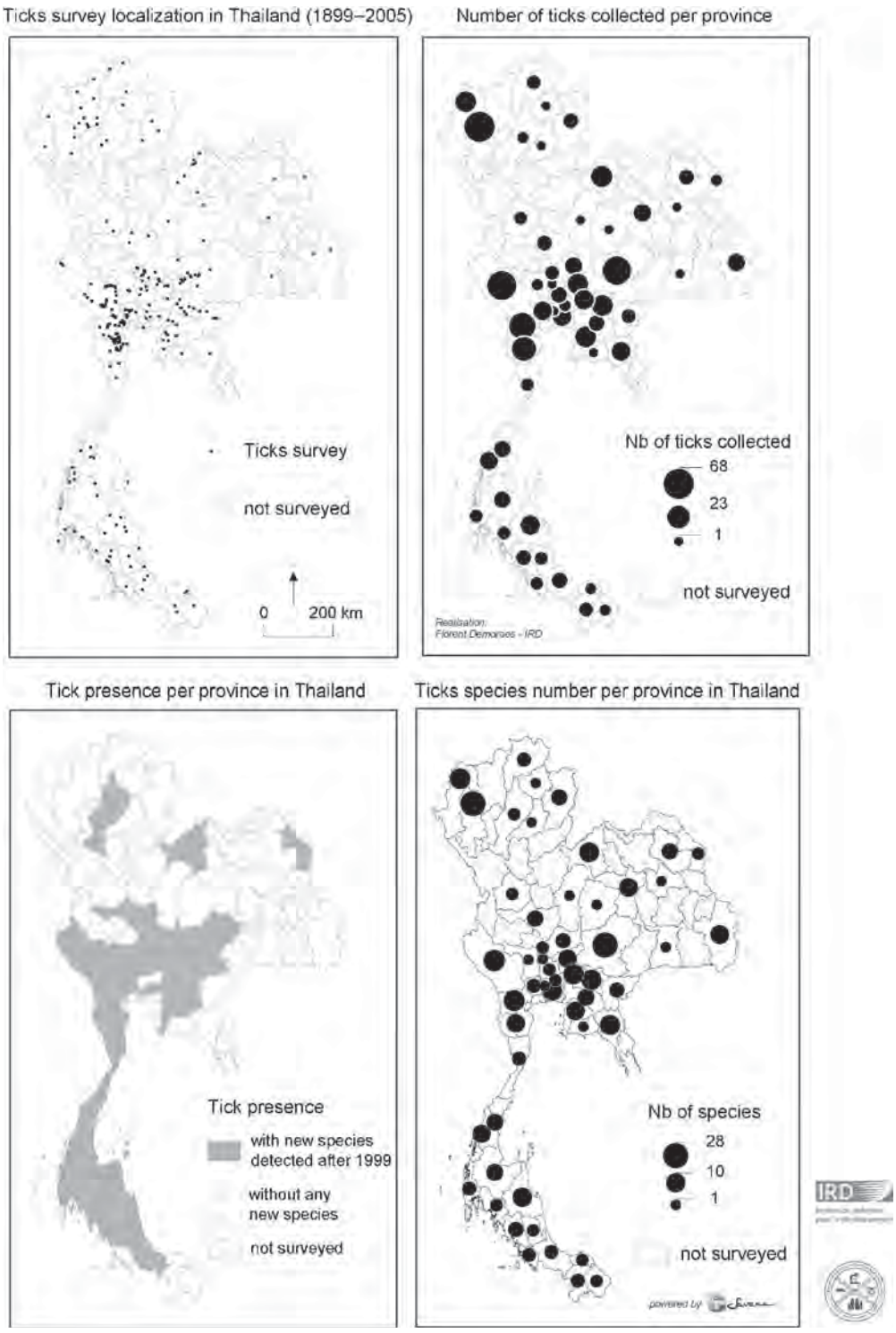

Fig. 32.12. Tick survey in Thailand. *Top* (left): Location of tick collection sites during the period 1999–2005 survey in Thailand ; (right) Absolute number of specimen collected by province (abundance). *Bottom* (left): Dark grey are the provinces where new tick species have been identified for the first time in Thailand; (right) the absolute number of species identified by province (biodiversity).

32.3.5.1.1 A homogeneous ecological framework

Since the discovery of Ebola virus in 1976, thirteen epidemic outbreaks (nine due to the subtype Ebola Zaire and four to Ebola Sudan) and two isolated cases (one due to Ebola Zaire and the other in Ebola Ivory Coast) have affected the African continent in three distinct periods: three outbreaks between 1976 and 1979, four between 1994 and 1997, and six between 2000 and 2004. On the whole, Ebola virus was

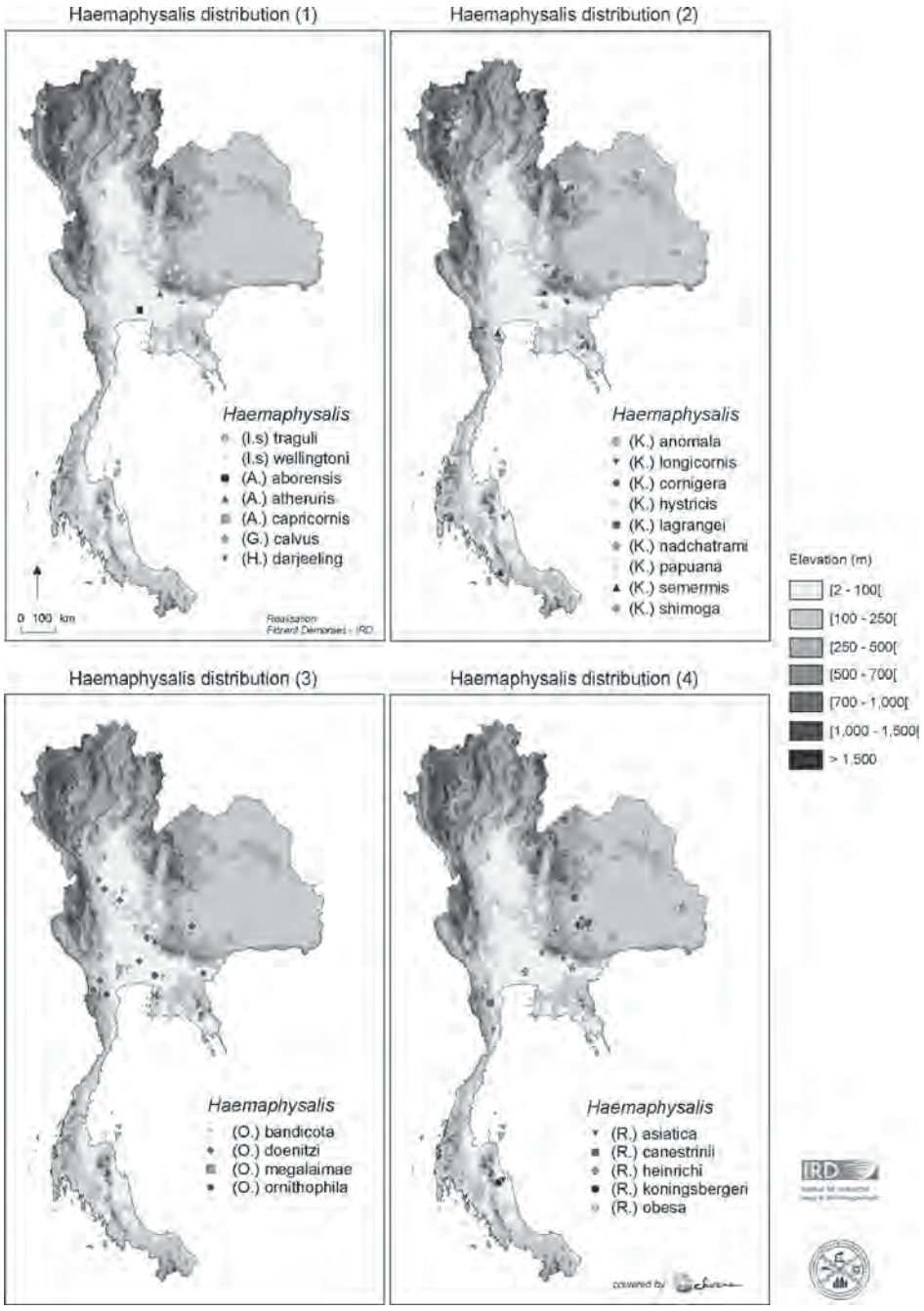

Fig. 32.13. Distribution of *Haemaphisalis* genus in Thailand. See color plates.

responsible for approximately 1850 clinical infections including nearly 1300 deaths. In addition, the descriptions show a tendency to a regional specificity of the subtypes of Ebola virus: Ebola Ivory Coast in West Africa [15,39]; Ebola Sudan in East Africa [3,17,31,42,61,72,74] and Ebola Zaire in Central Africa [30]. However, in spite of this relative geographical specificity, the ecological context of the human epidemics due to the African subtypes of the Ebola virus is relatively homogeneous and includes various forest areas located around the equator. These areas have similar ecological and climatic characteristics. The vegetation is dense, and the climate is tropical, hot and wet with two peaks of precipitation separated by two dry seasons.

32.3.5.1.2 The passage of the virus to the man In the majority of epidemics, the source of contamination of the

TABLE 32.3. Human Pathogen Arbovirus Isolated from Ticks in Central Asia, Siberia, and South East Asia

Virus Genus Type	Host Vertebrate	Vector Arthropod
Orbivirus		
Kemerovo	Bird	*Ixodes persulcatus*
Alphavirus		
Chikungunya	Bird, bat	*Argas* (I) *spp.* (2) (II)
Sindbis	Migratory bird, bat	*Hyalomma a. anatolicum, Ornithodoros savignyi (experimental)*
Flavivirus		
Karshi	Rodent	*Alectorobius capensis, Al. tholozan, Al. tar takovskyi, Hy. Asiaticum, Dermacentor marginatus*
Kyasanur forest disease	Rodent, bird, bat, primate	*Haemaphysalis sp (6), Ha. spinigera, Ha. turturis, Ha. wellingtoni, Ix. spp (2), Rhipicephalus spp (2), Hy. sp., De. sp. Al. spp (2), Ar. sp*
Langat	Rodent	*Ix. granulatus, Ix. Persulcatus, Ha. papuana*
Omsk hemorrhagic fever	Rodent	*De. marginatus, De. reticulatus, Ix. persulcatus*
Tick-borne encephalitis	Rodent, bird	*Ix. persulcatus, Ix. ricinus, De. marginatus, De. reticulatus, Ha. japonica douglasi, Ha. concinna*
Tyuleniy	Bird	*Ceratixodes uriae*
West Nile	Rodents, migratory bird, bat	*Ar. hermani, Hy. m. marginatum, Hy. asiaticum, Hy. Detritum, Ix. ricinus, De. Daghestanicus, De. marginatus, Rh. bursa, Rh. turanicus, Al. maritimus, Al. tholozani*
Nairovirus		
Crimean hemorrhagic fever	Rodent, cattle, goat, sheep	*Amblyomma variegatum, Hy. sp. Rh. sp*
Nairobi sheep disease	Goat, sheep	*Ha. intermedia, Ha. wellingtoni*
Bunyavirus		
Bakau	Primate, bird of prey	*Ar. abdussalami*
Bhanja	Rodents, cattle, goat, sheep shrew	*Ha. sp., Ha. intermedia, Ha. punctata, Hy. detri tum, Hy. m. marginatum, Hy. m. turanicum, Boophilus decoloratu, Rh. bursa, Rh. turanicus*
Issyk-Kul	Bat, bird	*Ar. vespertilionis*
Kaisodi	Bird	*Ha. spinigera, Ha. wellingtoni, Ha. turturis*
Lanjan	Rodent	*De. auratus, Ha. semermis, Ha. nadchatrami, Ix. granulatus*
Thogoto	Cattle, goat, sheep	*Rh. sp., Hy. anatolicum anatolicum*

first person identified as infected (first case) is unknown. It is the case for all epidemics of the period 1976–1979, of epidemics of Mékouka (Gabon) in 1995, of Booué (Gabon) in 1996, of Kikwit (RDC) in 1995, and of all the epidemics due to Ebola Sudan in 1976, 1979, and 2004 in Sudan and in 2000 in Uganda. Recent work however, allowed us to reveal that human contamination appeared these last years following the handling of infected carcasses of gorillas, chimpanzees, and duikers. Thus, it is probable that the carcasses of gorillas were at the origin of the transmission chains of Olloba 2001, Grand Etoumbi 2002, Entsiami 2002 and Yembelengoye 2002. Carcasses of chimpanzees were at the origin of the chains of Etakangaye 2001 and Olloba 2002. Lastly, carcasses of duikers were at the origin of the chains of Mendemba 2001, Ekata 2001, and Mvoula 2003.

32.3.5.1.3 The intermediate animal species sensitive to the virus At the time of epidemic episodes, which have occurred in Gabon and in RC between 2001 and 2004, many dead animals were found in the forest areas touched by the epidemics. On the whole, 44 carcasses were discovered, samples taken then analyzed at the laboratory of the CIRMF[11] between 2001 and 2005. On the 44 analyzed carcasses, 16 animals (12 gorillas, three chimpanzees and one duiker) were diagnosed positive for the infection by Ebola (Leroy, personal communication), which proves that these three animal species can be naturally infected by the Ebola virus. Calculations of indices of presence of the animals in certain places (excrements, paw prints, plants broken by animal passage, presence of nests, . . .) revealed a significant rise of mortality in certain animal species right before and during the human epidemics. The populations of gorillas and duikers seemed to have fallen by 50 % between 2002 and 2003 in the sanctuary of Lossi

[11]Centre International de Recherche Médicale de Franceville; *engl.*: International Medical Research Center of Franceville (Gabon).

Fig. 32.14. Tracking the deadly path of Ebola virus. The state of the carcasses allows for dating up to a few days from the death of the animal and to isolate the virus from tissue in sufficient state of preservation. *Top* (left): On the field sampling of cadavers and remains of animals potentially infected by Ebola virus during an epizooty of large monkeys (Gabon) Carcasses of large monkeys who died of Ebola virus infection: skull and hips (*top* right), hand and arm (*bottom* left), head in decomposition (*bottom* right).

(320 km^2), Republic of Congo, and those of chimpanzees by 88 %. Even if these results remain approximate insofar as one knows for example that the disappearance of an adult male gorilla dominating causes the bursting of the group and that the dispersed individuals are then difficult to count, they suggest nevertheless that the Ebola virus would appear to these animal populations in the form of great epidemics very localized in space causing the death of many animals in a very short period of time.

These results confirm certain studies indicating strong reductions in the gorilla and chimpanzee populations in the areas of Gabon touched by Ebola epidemics [27,67].

32.3.5.1.4 Infection of large monkeys The Ebola virus is a virus with very genetically stable ARN. For example, the mutation rate between the strains Booué 96 (Gabon) and Zaire 76 are only 1.7% for the glycoprotein membrane (GP), 1.3% for the nucleoprotein (NP), 1.2% for viral protein structure of 40 kDa (VP40) and 0.9% for viral protein structure of 24 kDa (VP24), whereas these strains were separated by more than 1000 km and spaced by 20 years [41]. Similar differences were found between genes in the epidemic strains of 2001–2003, the strain Mékouka 94 (Gabon) and Zaire 76. In the same way, sequencing of the most variable part of GP, bearing 249 nucleotides, did not show any change between nine patients (five convalescents and four deceased) taken at the time of the epidemic of Kikwit in Zaire (RDC) in 1995 [55].

In order to understand modes of contamination in large monkeys, studies based on this stability then amplified and sequenced the coding part of GP starting from samples taken from all gorilla and chimpanzee carcasses. These studies identified a different viral sequence for each analyzed carcass. Different sequences were obtained starting from the carcasses belonging to the same species (gorillas or chimpanzees) discovered at the same time at a few hundred meters from each other. The presence of many mutations between sequences obtained from the animal carcasses suggests that large monkeys were contaminated independently from each other. The presence of positive serologies detected in chimpanzees taken before the appearance of the first epidemic in this area confirms these conclusions.

It appears that Ebola epidemics in large monkeys result from massive and simultaneous contaminations of these primates starting with the animal reservoir and during particular environmental conditions (epidemics always occur at the same time of year, during transitional periods between the dry and rainy season). The contamination of humans is perhaps carried out in the second period, generally by contact with the animal corpses.

However, other authors explain the appearance of these epidemics differently. After mathematical modeling of various epidemic episodes carried out in Gabon and the Republic of Congo between 1995 and 2005 and also of coding sequences of GP for various viral strains, it is proposed that a displacement of an "epidemic wave" already existing for ten years according to a north–west, south–east movement and originating from Yambuku, town of RDC touched by the first epidemic of Ebola in 1976 [68]. This epidemic wave would then have affected the sensitive animal populations in a dramatic way at the time of its passage.

32.3.5.1.5 The reservoir of ebola virus In 2002 and 2003, studies include three animal trapping expeditions in two forest belts affected by the various epidemics, which have occurred between 2001 and 2005. The captures were carried out in a radius of 10 km around a gorilla carcass infected by Ebola virus, during a period of three weeks, and began only a few days after the discovery of the carcass. Conditions were met so that the captures were made at the height of virus circulation in its natural environment. A total of 1030 animals were captured, autopsied, and analyzed over a period of 4 years. These analyses showed that three species of frugivorous bats were asymptomatically infected by the Ebola virus: *Hypsignathus monstrosus*, *Epomops franqueti*, and *Myonycteris torquata*. Thus, anti-Ebola IgG were detected in serum of 16 bats including four *Hypsignathus*, eight *Epomops*, and four *Myonycteris* whereas they were found in no other bat or animal species. In addition, viral nucleotidic sequences were detected in the bodies of 13 bats including three *Hypsignathus*, five *Epomops*, and five *Myonycteris*. The sequencing of amplified fragments confirmed the specificity of sequences. The phylogenic analysis of these sequences shows that they belong to the subtype of the Ebola virus found in Zaire. Even if this research did not achieve virus isolation, it constitutes the first biological evidence for identifying certain frugivorous bat species as Ebola virus reservoirs. In addition, these results agree with species distribution covering the epidemic areas [4].

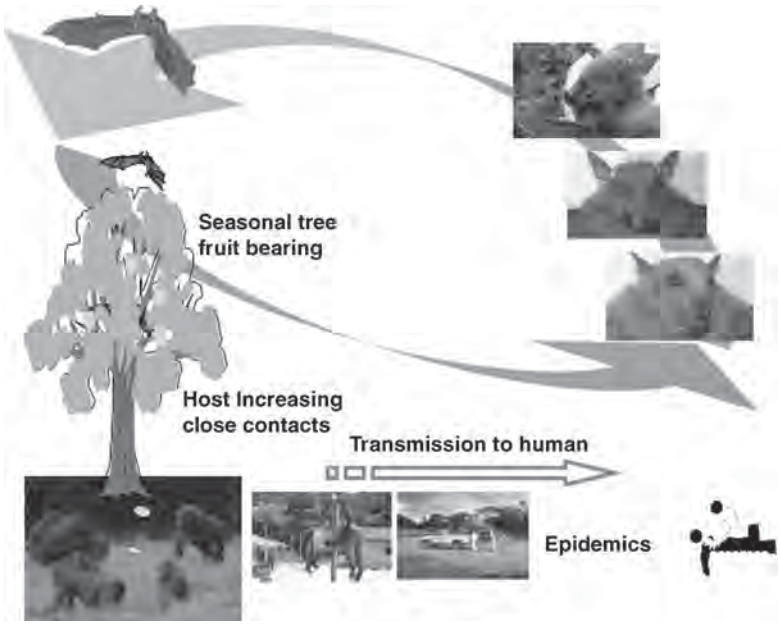

Fig. 32.15. The complex and hidden natural cycle of Ebola virus: a variety of hosts, overlapping domains and an elusive virus reservoir. The three bat species considered as Ebola virus Reservoir Host in Central Africa (picture on right arrow of the cycle and from top to bottom), are *Hypsignathus monstrosus* (hammer-headed fruit bat), *Epomops franqueti* (singing fruit bat), *Myonycteris torquata* (little collared fruit bat), are frugivorous, live in the tropical rain forest of equatorial Africa, they are not migratory species.

This discovery consolidates certain epidemiologic indices collected at the time of the epidemics due to the virus of Marburg, another member of *Filoviridae*. Many bats were located on several occasions in warehouses of the cotton factory where the first patients of the epidemics worked in Nzara, Sudan, during 1976 and 1979. No source of contamination having been identified for these two epidemics, a contamination by bats in the cotton factory has been considered.

The Australian case of Marburg who contaminated two other people at Johannesburg in 1975, revealed that he had just returned from a voyage to Zimbabwe during which he had slept out doors and once in an abandoned house whose attic was inhabited by many bats [16]. The French engineer, infected by the virus of Marburg in Kenya in 1980 and responsible for the contamination of the doctor who had treated him, had, a few days before becoming sick, visited several caves inhabited by significant populations of bats [62]. In order to explore the assumption of the contamination by the bats within these caves, caged baboons and vervets were placed inside these caves. But none of them became sick (Johnson, personal communication, 1996).

The characterization of nucleotidic sequences in Ebola genes, which codes the ARN polymerase (protein L) and the nucleoprotein (NP) highlighted significant areas homologous with equivalent genes of *Paramyxoviridae* and *Rhabdoviridae* [48,60] reinforcing the genetic relationship of these two viral families with *Filoviridae*. Several studies evoke the possibility of direct contamination of humans by some of these viruses originating in bats. As an example, the viruses Hendra [24] and Nipah [29] could be directly transmitted to the humans by the saliva of frugivorous bats deposited on fruits [44]. In the same way, certain variable of the rabies virus would be transmitted directly to the man by insectivorous bites of bats [13, 19].

Lastly, certain species of chiropteran (of the genus *Epomophorus* and *Tadarida*) developed a transitory viremia lasting four weeks after intravenous inoculation, suggesting that the Ebola virus can infect bats without developing disease [63]. Certain observations suppose that the contamination of the large monkeys would intervene in favor of direct contacts with blood and/or placental tissue of the females at the time of parturition. The epidemiologic field surveys carried out showed that large monkeys died from Ebola virus at the end of the dry season. Food resources would be scarcer during the dry season in the tropical forests of Africa, which would cause various frugivorous animal species to move at the same place. This promiscuity would support the contacts between large monkeys and bats and thus the viral transmissions of the virus between the two animal species.

32.3.5.2 Avifauna and infectious diseases Migratory and non migratory birds take part in maintaining pathogenic agents responsible for infectious disease transmission to humans; they act as vector or intermediate host for germs which, for the bird itself, may be pathogenic (e.g., Psittacosis) or not (e.g., West Nile). What interests the particular field of disease emergence, is less in the role of vector-host that birds

play in germ transmission to humans but rather more their role as "carrier" transporting the germ (or an ectoparasite vector of a germ) beyond the normal fields of disease circulation and being able to play a significant role in pathogenic virus dispersion. If this role seems obvious to explain geographical emergence of certain diseases it still remains badly known, and little studied (e.g., Bird Flu; virus West Nile). This is due to the immense variety of avifauna, the difficulty in making representative biological samplings, the mobility of these hosts, the exchanges of germs, which can be done in rest areas (stop over, roosting sites) of migrating species. Recent events in bird associated pathogen emergence, have lent topicality to the question of the bird's role in virus transport and research in this field has beneficed from technological progress and accumulated knowledge on avian fauna in all continents.

Bird behavior is largely influenced by environment particularly climate in relation to vegetation and abundance of food and also human behavior which often displaces wild bird's habitats to exploit the land or by regrouping domestic birds for breeding and exploitation.

In this chapter we will focus on the role of birds as virus vector within the framework of dispersion. Two exemplary virus diseases come to mind: the West Nile virus encephalitis and avian and swine flu. For these two types of virus, the birds can infect themselves and develop a more or less severe clinical picture or be a healthy virus carrier.

Fig. 32.16. Tracking birds for a follow up on their potential role of pathogen dispersion. *Top to bottom*: Biological sampling (blood, swabbing) an Ombrette in Burkina Faso; storks ringed with four solar transmitters Argos—GPS with their antenna visible ready to be equipped; the transmitter in place on the back of bird ready to be set free. *Background map*: Migration route of Black Stork (2004–2005); red line: Aurélia: Migration outward journey (April–June 2004); orange line: Aurélia: Migration Return (July–September 2004); green line: Camille: Migration outward journey (August–October 2005). *Left to right, from top to bottom*: (A) biological sampling (blood, swabbing) on a Hamerkop in Burkina Faso; (B) Young Storks captured to be ringed and equipped with GPS Argos solar transmitters; (C) Young Storks to be ringed, transmitters with their antenna are visible next to birds ready to be equipped. (C) Argos GPS solar transmitter placed on bird's back, ready to release bird. See color plates.

32.3.5.2.1 Where do birds and their viruses migrate? Tracking birds to determine their potential role in pathogen dispersion[12] The current use of satellite radio transmitter for following wild fauna opens many new ways of exploring and understanding animal behavior, its strategies of reproduction of alimentation, the degree of adaptation to its environment or the seasonal migration of these species. Coupled with geographical, field data, these techniques of localization by satellite reception provide incomparable information, in real time, on the occupation of space or the exploitation of natural resources.

For the migrating avifauna, nesting in the palearctic region and wintering in West Africa, the first experiments on migratory birds by satellite localization go back ten years but involved only battery powered transmitter, without built-in geopositioning by satellite (GPS), not very precise but nevertheless useful because they made it possible to specify the axes of migrations of certain species. Today, it is possible to determine the migrations with a precision of a few dozen meters, and to post the results almost on line on the screens of the microcomputers of users of this service.

In this context, IRD ornithologist researchers, developed since 2004, scientific work on the winter ecoethology of *Ciconia nigra* starting from Burkina Faso by using a 70g solar transmitter, equipped with a GPS system (Argos© type). This research is completely innovative as regards follow-up of avifauna in sub-Saharan Africa and constitutes a pilot study for follow-up of migrating birds of medical interest (as potential vectors), and also the immunological follow-up of this migration by capture/release to obtain a sample (20 µl blood) necessary for a serology of micro-method.

These techniques of high precision, coupled with multiple ecological readings from the field over a period of 2 years made it possible to define and model; the trophic routes, requirements, and daily activities of the followed individuals (hours of food, hours of rest, azimuth of take-off, altitude, rate of travel, roosting place, etc.), and also to measure competition for territory and food between *Ciconia nigra* and a local group of fishermen. The initial samples were negative in specific immunoglobulins of the studied viruses.

The acquisition of this type of data obtained from a dynamic biological model such as *Ciconia nigra*, sharing with human populations the same food resources, geographical space and a common seasonality opens multiple prospects for transdisciplinary research, in particular for the study of avifauna potential reservoirs of pathogenic agent to humans. The sporadic appearance of the West Nile virus in Europe and on the African continent constitutes, in this respect, a relevant model. The principal hosts of this virus are birds both domestic (ducks, pigeons), or wild and probably play a fundamental role in the dissemination of the virus between the Palaearctic field and the African continent, during the pre or postbreeding migrations. There, mosquitoes (of the genus *Aedes sp.* and *Culex* sp.) are infected when blood feeding on these birds and locally maintains the cycle mosquitoes/birds, necessary to the circulation of the virus. In this context, the use of solar transmitter equipped with GPS, posed on potential host birds (species found seropositive) could provide invaluable information on the spatial and temporal dynamics of this avifauna and, in consequence, on that of the cycle of viral agents which they can carry. The role of soft ticks, common ectoparasites in birds and vector of pathogenic viruses could also be studied by targeting species of birds of medical interest.

32.3.5.2.2 Highly pathogenic avian influenza (Hpai) emergence in Thailand: the spatial aspect of emergence and the dynamics of an epizooty In 1997, the appearance in Hong Kong of human cases of influenza related to an avian influenza caught the world's attention and made influenza a central public health care issue in many countries. Virus A of influenza always presents a significant potential danger to human populations, by its effective mode of transmission in the event of promiscuity, by its mutation capacities and genetic reassortment, which prevent installation of an effective natural immunization in human populations, and by the existence of an enormous and badly defined natural reservoir (in particular the many wild migrating birds), which contributes to maintain its genetic diversity. Strain H5N1 had been isolated on several occasions since 1959 on birds, in the United Kingdom (1959, 1991), in the United States (1975, 1983), and in China (1996), but without causing epidemics in the birds and especially without causing human cases (all the viral strains of H5 type are avian and all the H5Nx combinations were already isolated on birds , except H5N4 none had ever caused human cases). The avian viruses develop primarily in the digestive tract, contrary to the human viruses, which touch initially the respiratory tract, the receptor of the hemagglutinin being different according to species.

The H5N1 virus emerged in Hong Kong with a very significant pathogenic capacity for chickens, and the capacity to infect humans directly with a death rate of 30 %, well beyond 2.8 % of the strain type H1N1 responsible for the 1918 Spanish influenza pandemic. But the virus infected humans only in a very marginal way, and was unable to be transmitted directly from person to person, a quality, which is still not acquired in 2005. These marginal infections (18 cases in Hong Kong in 1997, six of it died) nevertheless created fear of a generalized human diffusion in the event of genetic viral modification (the hemagglutinin induces a highly protective humoral response in humans, but antibodies corresponding to the H5 antigen do not exist in humans). Fearing the worst, the Hong Kong authorities had then taken drastic measures to try to eliminate the virus from the peninsula, by ordering the destruction of all birds, whether they were wild, or domestic (1.5 million birds). Actually eradicated in 1997 by these measures, the virus nevertheless since reappeared on a small scale in 1998 (China, Hong Kong), then more widely since 2003 (China, Japan, Korea, Indonesia, Vietnam, Laos, Kampuchea, Thailand), to extend unrelentingly in a large part of South Asia from 2003 to 2005, then progressing slowly toward the west to Eurasia and Europe in 2005 and Africa in 2006.

[12]François Baillon and Damien Chevallier, IRD—C.N.R.S, unpublished data.

TABLE 32.4. History of Avian Influenza in Hong- Kong Since 1997

March/April 1997	Avian flu outbreak among chicken farms in northwestern part of Hong Kong
9.5.1997	Onset of illness for the first case of influenza A (H5N1)
18.8.1997	Laboratory confirmation of H5N1 infection for the first case
26.11.1997	Confirmation of the second case of human infection.
December 1997	Isolation of H5N1 virus for chicken markets. More human cases
23.12.1997	Poultry export from Mainland suspended at midnight
28.12.1997	Evidence of widespread H5N1 infection in a chicken farm and wholesale market
29.12.1997	Slaughtering of chickens and poultry commenced. No new human infections occurred since then in Hong Kong

Among great pandemics of twentieth century, the influenza has been greatly studied, the pathogen is well known, surveillance networks exist in many countries, the recommendations of FAO, OIE, and OMS largely diffused, but the emergence of a new viral strain potentially very pathogenic for humans reveals the many questions that remain related to emergence, and which make influenza an exemplary and always topical disease by the danger which it represents. The control of the influenza is never a local question, but too many countries do not yet adopt the recommendations of international organizations, and sometimes several months are necessary before the emergence of an epizooty alerts political attention, whereas speed of action is essential to contain the geographical expansion of the disease, whether it is human or avian. In Hong Kong, the response of the authorities was fast and effective as soon as human cases were detected, and made it possible to remove epizooty and the epidemic. But the many studies which had been led in Hong Kong really did not make it possible to understand the emergence mechanisms and the progression of the disease toward other areas of the continent could not be contained, more especially as it reached countries where the monitoring and control do not have the desired effectiveness.

In the case of avian influenza, the questions are numerous: Is the reservoir badly defined or too broad to be understood (wild birds or/and mammals)? The initial vector is badly known (migratory birds)? What is the mode of diffusion, displacement of wild birds, poultry transport, or other mammals? Human practices? Cause of seasonality and disappearance period is unknown (Migrations of birds? Climatic conditions?). We will highlight these questions by studying the epizooty of avian influenza in Thailand since 2003. Thailand, large poultry producer and exporter, had made an exemplary step in the management of this crisis (delay in political awareness, delay in initial detection of the disease, usual propaganda—chicken meat tasting—but at same time active veterinary monitoring, data collection faltering at the beginning but quickly effective, monitoring of wild birds and fauna, research of reservoirs and infected nonsick animals, strict application of the recommendations of OIE, etc.).

32.3.5.2.3 Avian influenza in Thailand since 2003 Thailand was reached by avian influenza of the strain H5N1 at the end of 2003. Then, after the disappearance of epizooty by May 2004, the disease was recorded again, as from July 2004 and in 2005, according to a regular and seasonal cycle. Probably thanks to the medical measurements applied strictly since November 2004, but perhaps also by a reduction in the virulence of the stock, the third wave of epizooty (2005) was much weaker.

32.3.5.2.4 The first wave, January–April 2004 After China and Hong Kong, the avian influenza due to virus H5N1 was detected at the beginning of 2004 simultaneously in Thailand, in Laos, and in Kampuchea, after having been reported from Korea and Vietnam (December 2003), Japan and Taiwan (at the beginning of January 2004). It then reached Indonesia in February. Surprisingly, certain countries did not report any cases (Burma, Malaysia).

Avian influenza most probably appeared in Thailand around the middle of 2003 without being immediately reported. The first veterinary report dates from January 23, 2004 (Suphanburi province, eggs producing farm) and then cases in poultry farms

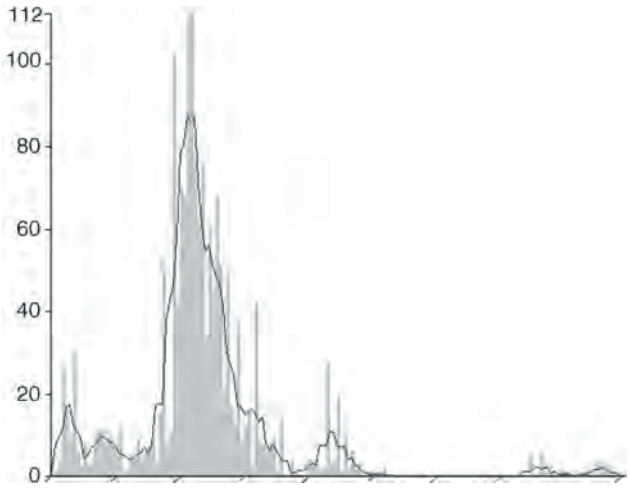

Fig. 32.17. Avian flu Histogram of reported positive foci by district previously not infected from July 2004 to November 2005. The *X*-axes numbers indicate the number of elapsed weeks starting from July 2004. The *Y*-axes values are given by the number of foci reported positive by week. All data presented come from reports of cases focus carried out by the DLD (Department of Livestock Development, Ministry of Agriculture, Thailand). The data are mapped by district. Thailand comprises 921 districts, which are the intermediate administrative divisions between provinces and the local level.

followed one after another very quickly (in the last week of January 2004, the disease was reported 157 times in 36 provinces in Thailand). Meanwhile in Thailand, the human cases preceded the declaration of avian cases (first human case on January 3, 2004 in the province of Suphanburi, a total nine cases in January, including seven deaths). In February, the last human cases of this first wave were detected (three cases including two deaths), whereas the avian influenza was reported 22 times in 12 provinces, in March 6 times in five provinces, in April 4 times in three provinces, in May only once. No case was recorded between May 24 and July 3, 2004, beginning of the second wave. In poultry farms, this first wave reached primarily the chickens (60 %), the distribution by species being virtually identical to that of the epidemic of Hong Kong in 1997. The human cases were always related to the presence of an avian case.

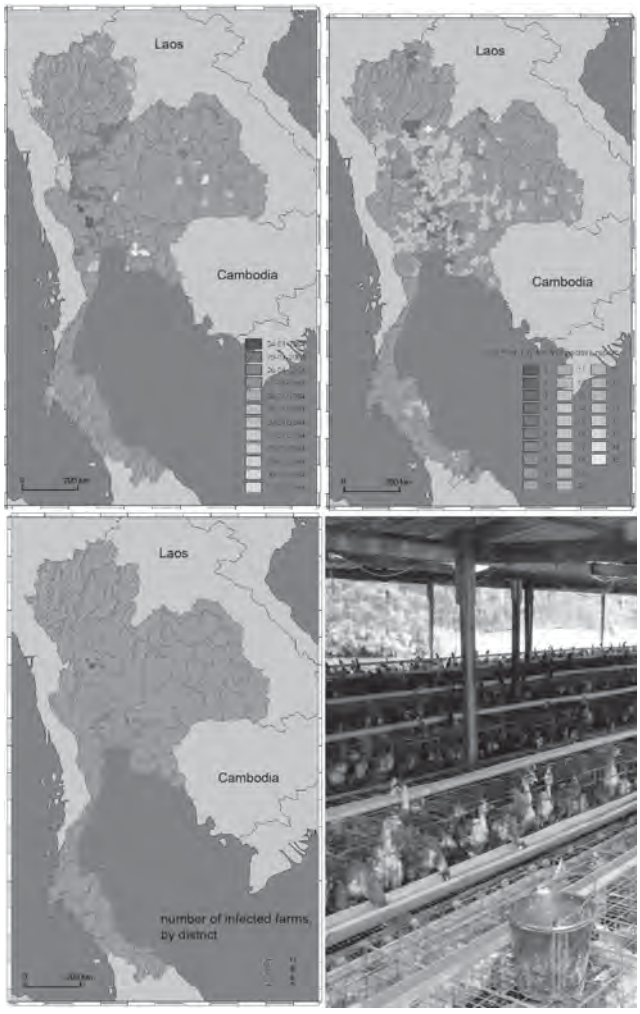

Fig. 32.18. Highly pathogenic avian influenza in Thailand. *Top left*: Geographic expansion of first wave of H5N1 epizootic in Thailand (January–February 2004) reported by day and by district. *Top right*: Geographic expansion of second wave of H5N1 epizootic in Thailand (July–April 2005) reported by week and by district. *Bottom left*: Number of foci reported by week and by district during the third wave of H5N1 epizootic in Thailand (July 2005–November 2005). *Bottom right*: A poultry farm from the Samut Sakhorn province, Thailand. See color plates.

At the time of the first wave of expansion in 2004 (January 2004–May 2004), information was not clearly disseminated, certain countries denying quickly any infection, others maintaining an opacity related to the economic importance of the sector's poultry industry. It is only during the second wave of epidemic (July 2004–February 2005 in Thailand) that the authorities adopted a certain transparency, in particular in Thailand. Once installed, medical measures were quickly effective in stopping the diffusion of epizooty, but it is probable that the seasonal character of epizooty supported the fast eradication in Thailand. The country was declared free from avian influenza in May 2004.

32.3.5.2.5 The second wave: July 2004–April 2005 In Thailand, the second wave begins in July 2004 and persists until April 2005, with a significant peak in October–November. The reports of cases are now much better organized, laboratory tests are carried out systematically by the DLD (Department of Livestock Development, Ministry of Agriculture, Thailand), and main information is available very quickly on the DLD Website.

The reported cases are much more numerous than during the first wave (1669 cases tested positive). Many monitoring tests are carried out on the domestic animals and on wild fauna (migratory birds, rodents). In November, the government "declares the war with epizooty," carries out active and passive monitoring, takes control measures aiming to eradicate the disease in the short term and releases significant funds (National Strategic Plan and Avian Influenza and Plan for Pandemic Preparedness, 2005–2007, USD 105 million).

The emergence of the first cases is located in the zone, which had already seen the appearance of the first detected cases at the beginning of 2004. The geographical expansion seems to show initial cases in the north of the central plain, then a local extension starting from these cases. The initial cases do not correspond to the most active zones of industrial avian production, but rather to ecological zones favorable to the natural reservoir. However, the role of wild birds in local or national diffusion was not highlighted. The human practices and behaviors (transport of poultries, markets, cockfights) are also probable causes of the diffusion by proximity. The domestic chickens were the most hit by epizooty (57 %), then the ducks (29 %), the industrial chicken farms (5 %), and the farms producing eggs (5 %). The cases relate primarily to farms where the number of birds is modest (the median is 75). Culling is systematic in farms as soon as several birds are found dead (10 % of farms), and measures of containment are applied during 21 days (no displacement of poultry for all exploitations in a radius of 5 km around the infected farm).

Reemergence after a silent period leads to three assumptions: either the virus circulates asymptomatically in certain reservoir animals, or it is maintained in the environment in water), or seasonal migratory birds reintroduce it.

TABLE 32.5. Origin of Avian Flu Human Infections in Thailand, 2004

No.	Sex	Age (year)	Address	Onset	Hospital	Diagnosis	Outcome
1	M	18	Tambon Khao Maikaew, Kabinburi district, Prachinburi	August 31, 2004	Kabinburi	Pneumonia	Died, September 8, 2004
2	F	26	Tambon Pang Maka, Khanuworalakburi district, Kamphaeng Phet*	September 11, 2004	Pakkretvejakan	Pneumonia	Died, September 20, 2004
3	F	32	Tambon Pang Maka, Khanuworalakburi district, Kamphaeng Phet	September 16, 2004	Kamphaeng Phet	Pneumonia	Discharged, October 8, 2004
4	F	9	Tambon Budhabat, Chondaen district, Phetchabun	September 23, 2004	Chondaen and referred to Phetchabun Hospital	Pneumonia	Died, October 3, 2004
5	F	14	Tambon Pa-ngew, Srisachanalai district, Sukhothai	October 8, 2004	Srisangworn	Pneumonia	Died, October 19, 2004

No cases were notified between April 12 and July 1, 2005. The third epidemic wave begins in the province of Suphanburi, where the epizooty was detected for the first time in Thailand. From July 1 to November 15, 75 cases were reported: Epizooty was thus much less active, either because of surveillance and control measures or by a reduction in virulence. This reduction, which was reported in certain articles, is not confirmed by the observations by the DLD (average mortality of 95 %), but these results are difficult to interpret (many reports of cases do not indicate the number of dead or sick birds).

32.3.5.2.6 The factors of emergence and diffusion The migratory birds were immediately blamed as the factor of emergence for avian influenza in Thailand, with no evidence for this thesis. Indeed, some colonies of storks (Open bill stork) present in Thailand migrate from Bangladesh, and the mortality of these birds increased considerably in 2004, but as no case of avian influenza was listed in Bangladesh: It is more than probable that these birds were more victims than responsible for epizooty.

Many wild birds perform local migrations (in particular wild ducks, which mix with domestic farm birds), and can be responsible for local diffusion. Natural and artificial ponds are very numerous in Thailand, and open-air chicken or ducks farm are frequent particular farmyard chickens.

The diffusion of epizooty according to a north–south axis, which follows the large road network in Thailand, can also let us think of a diffusion related to transport related to poultry industry. Very restrictive measures of limitation of displacements of poultry, with more than 150 points of control between areas, were taken since 2004 on this assumption. The practice of cockfights was also blamed like a factor of significant risk in the diffusion of the disease in animals.

A silent period, from April to July, has been noted for 2 years. The viral flu can be probably maintained several weeks in cold water, but this period corresponds to a very hot weather in Thailand.

32.3.6.1 Rodents and virus: how a viral family can have a global distribution? The rodent vectors of virus to humans can be represented by two exemplary systems, the "Rodents and Hantavirus" model and the "Rodents and Arenavirus" model. In both cases, there is a narrow association between a type of virus and a species of rodents. With the development of compared phylogenetic analysis of the hosts-vectors, it appeared clearly that the rodent–virus couples had followed an evolutionary process in the form of coevolution and of cospeciation. What was in the years 80s still a hypoth-

Fig. 32.19. H5N1 infected Open bill stork dying in the natural resting area. The agonizing stork showed in the pictures has probably been infected by nearby poultry during the second epizootic of H5N1 in Thailand.

TABLE 32.6. The Arenavirus, Their Hosts and Emergence Domains

Acronym	Virus	Country and Date[a] of Emergence	Virus Reservoir
Old World Arenaviruses			
LASV	Lassa	Nigeria, 1970	*Mastomys huberti*
MOBV	Mobala	Central African Republic, 1982	*Praomys sp.*
MOPV[4]	Mopeia	Mozambique, 1977	*Mastomys natalensis*
IPPYV	Ippy	Central African Republic, 1978	*Arvicanthus niloticus*
LCMV	Lymphocytic Choriomeningtis	USA, 1934	*Mus musculus*
New World Arenaviruses (North Central America)			
BCNV	Bear Canyon	USA, California, 1966	*Peromyscus californicus.*
TAMV	Tamiami	USA, Florida Everglades, 1970	*Sigmodon hispidus*
WWAV	White Water Arroyo	Southwestern USA, 1996	*Neotoma albigula*
New World Arenaviruses (South America)			
Lineage A			
ALLV[5]	Allpahuayto	Peru, 2001	*Oecomys bicolor*
FLEV	Flexal	Brazil, Amazonia, 1977	*Oryzomys capito*
PARV	Parana	Paraguay, 1970	*Oryzomys buccinatus*
PICV	Pichinde	Colombia, 1971	*Oryzomys albigularis*
PIRV	Pirital	Venezuela, 1997	*Sigmodon alstoni*
Lineage B			
AMAV	Amapri	Brazil, 1966	*Oryzomys capito*
CPXV	Cupixi	Brazil, North Easter, 2002n	*Oryzomys capito*
JUNV	Junin	Argentina, 1958	*Calomys musculinus*
GTOV	Guanarito	Venezuela, 1991	*Zygodontomys brevicauda*
MACV	Machupo	Bolivia, 1965	*Calomys callosus*
SABV	Sabia	Brazil, 1994	Unknown
TCRV	Tacaribe	Trinidad, 1963	*Artibeus spp.* (bat)
Lineage C			
LATV	Latino	Bolivia, 1973	*Calomys callosus*
OLVV	Olivero	Argentina, 1996	*Bolomys obscurus*

[a] Date of emergence and/or publication.

esis became a dogma with the emergence of new virus taxa in two models [5, 9, 20, 21]. The process of coevolution was modulated by phenomena of disappearance of one of the partners, or the discovery of hosts without virus or a virus taxon without known host. Finally, after a second review of host phylogeny and of their known virus it is possible to predict the presence of an undeclared virus in a domain where potential hosts are present: Thus, like the "Hantavirus in Africa" suspected by the demonstration of serologic markers, without viral insolation, "the Arenavirus of Asia" also remains to discover. Let us see through the history of Murids how could Arenaviridae be spread to understand the vast domains these two families colonize world-wide in the present day.

32.3.6.1.1 The rodent: a brief history[13] Because it is often mentioned in literature, the term of "Murid" corresponds to the *Murinae* subfamily [Musser and Carleton, 1993 in 21]. What it is called Cricetids, needs to be specified as "New World Cricetids" including the New World rat and mice or the *Sigmodontinae* subfamily.

We use the most common theory on rodent radiation to support part of our hypothesis. From the Eurasian continent, the Cricetids, ancestors of the Murids, spread into the Americas, and then, from Asia, the Murids spread to Europe and Africa.

As early as the Eocene, 65 millions years before present (My.B.P.), within North America, a rodent ancestor, *Simimys*, is found bearing Cricetid characteristics. During the Oligocene (37 My.B.P.), the Cricetid repartition became holoartic. The New World Cricetids colonized by waves of migration northward and southward to the Americas. As a result, the Cricetid fauna from South America is coming from North America and today; it appears that the group from South America can be separated to the less diversified group of *Neotoma-Peromyscus* from North America.

[13] Adapted and revised from, Gonzalez JP, Arenavirus and Hantavirus and rodents co-evolution. Turk *Arch Parasitol*, 1996.

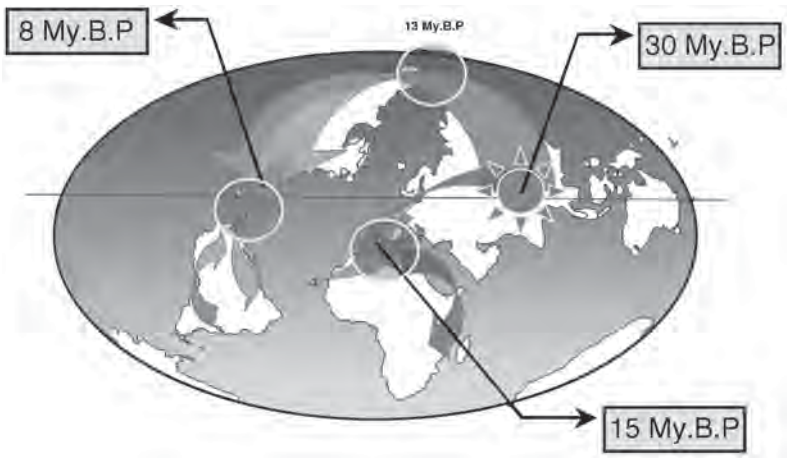

Fig. 32.20. The rodent dispersion from an Asian origin.

In Asia, the Cricetids came more likely from North America and were present during the Oligocene (35 My.B.P.). Although, from an original pool, and by successive waves, Cricetids spread to Europe during the late Miocene (15 My.B.P.), they became underrepresented in Africa with a limited extension.

From Asia, 14 My.B.P. Murid spread around the Mediterranean basin to Europe. During that period, *Murinae* extend from Europe to North Africa and rapidly became the most widely spread rodents in Africa.

Beginning the Pleistocene (2 My.B.P.), Murids are present in sub-Saharian Africa. Then they largely spread from the north and south, while severely influenced in their radiation by arid climate and geomorphology. During that time, speciation became at its highest point influenced by climate changes and physical isolations: the Rift Valley and the Sahara divide the African continent. More recently, humans must have played an important rule for the spread of rodents and mostly with the comensal species. Some genuses from the Pleistocene are still present in East Africa, whereas others from North Africa disappeared. However, it likely that Murid ancestors were very closely related to the present genus.

In conclusion one can consider that the arenaviruses in general had as a principal mechanism of evolution a narrow process of coevolution and cospeciation with their reservoir the rodents. The dispersion of the arenaviruses and their diversification followed those of the rodent hosts. The emergence of strains pathogen for humans remains entirely misunderstood, undoubtedly due to the rarity and specificity of emergence conditions. As the encounters of these vectors with humans increased in a context of exploration with growing human populations, the probability of meeting an infected rodent also increased. On the Asian continent, even if there is evidence in South East Asia of their circulation, the discovery of the Arenaviruses still remains elusive. It is the same in Australia where only the Lymphocytic Choriomeningitis virus of mice (LCMV) has been announced in a laboratory mice-breeding center. These viruses, which remain to be identified, would certainly give additional elements to the

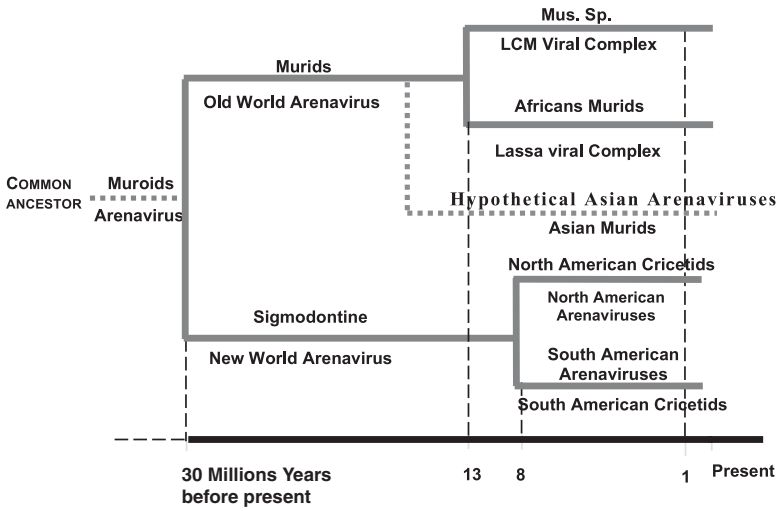

Fig. 32.21. A putative coevolution of Asian Murids and their arenavirus parasite.

coevolution of the rodents and their virus having migrated very early from Asia then toward Australia.

Above and below each horizontal line appear the main murid group and its corresponding arenavirus in a coevolution pattern. A specific arenavirus of Asian rodents has not yet been discovered and a putative new clade of "Asian arenavirus" is speculated in the present figure. This diagram represents large groups of viruses and their rodent hosts with which they have coevolved. In Asian rodents, among the oldest no arenavirus has yet been identified, a new taxon is proposed but remains to be identified.

32.3.6.2 From wildlife to humans: the SARS or the risk of early domestication process

32.3.6.2.1 The SARS outbreak or the first emerging disease of the twenty-first century First epidemic emergent disease, which marks the twenty-first century, the SARS (Severe Acute Respiratory Syndrome) diffused around the world in a few months. The principal mechanism of this fast planetary dispersion resides primarily in the displacement of infected people on airways. In spite of the great number of cases (8096 cases counted for 28 countries during the preepidemic and epidemic periods, and the strong mortality (9.6 %; that is to say 774 reported deaths) associated with this Severe Acute Respiratory Syndrome, the disease is eradicated relatively quickly. Less than four months after its isolation and its molecular characterization (mid-March 2003), the virus diffusion is restricted, thanks to simple protection measures of the individuals at risk and by insulation of the patients, new infections became sporadic with the last case reported in early September 2003. The ecology, origin, and evolutionary history of this virus remain to be established.

32.3.6.2.2 From a chinese market to the toronto airport: the lessons of SARS On November 16, 2002, a respiratory syndrome, associated to myalgias and a strong fever is reported in populations of the province of Guangdong, in South China. The data is missing to determine with certainty the number of cases before and after this period undoubtedly preepidemic. The Chinese ministry for health communicates its concerns with respect to this epidemic starting from the mid-February 2003 and announces, at the end of February, that the probable responsible agent would be the bacterium *Chlamydiae pneumoniae*. No other official source confirms this assumption.

The epidemic extends then out of China with two particularly remarkable initial cases: A 65-year-old doctor, residing in a Hong Kong hotel, and a Vietnamese in his 50s, admitted to Hanoi hospital at the beginning of March. These two patients present in turn the symptoms of disease and demonstrate the facility with which the infectious agent carries out inter-human transmission. The Hong Kong hotel counts 12 additional infections, among residents and visitors, in the hours, which follow the diagnosis of the 65-year-old doctor. Still in Hong Kong, in the Prince of Wales Hospital, only a few hours later 18 members of the personnel declared themselves sick, more than 50 employees of this hospital develop a fever accompanied by respiratory symptoms. Similarly, in Hanoi, hospital counts 20 contaminations among its medical personnel; mainly nurses, doctors, and surgeons constitute the victims of this epidemic. The hospital environment seems to have become a privileged medium in which the epidemic of Severe Acute Respiratory Syndrome (SARS, this name was given on March 10, 2003) makes most of its victims.

At the end of March, researchers of Hong Kong, the United States, and Germany identify the etiologic agent responsible for SARS [34, 52, 54, 57]. This new *Coronavirus* (order: *Nidovirales*), is unknown and named *Severe acute respiratory syndrome Coronavirus* or [SARS-CoV]. Whereas knowledge relating to this new *Coronavirus* increases exceptionally quickly thanks to an international scientific collaboration without precedent, the epidemic progresses and touches more and more cities in Asia (Hanoi, Hong Kong, Singapore) and in North America (Toronto). At the end April, governmental measures are taken in particular in China where the regrouping of populations is prohibited [75].

May 23, 2003, two teams of Hong Kong and Shenzhen detect a *Coronavirus* in wild animals collected in markets in the south of China. Species tested positive: the masked palm civet (*Paguma larvata* Smith, 1827), the burmese ferret-badger (*Melogale personata* Geoffroy Saint-Hilaire, 1831) and the racoon-dog (*Nyctereutes procyonoides* Gray, 1834; only serological evidence), belong to the Carnivora order and carry several coronaviruses, some being very close to [SARS-CoV].

Civet appears in the menu of many hotel establishments and is a revered delicacy of southern China. Culturally, the consumption of civet is well established in the area of Guangdong and Li Shizhen describes wild civet as invaluable in his "Great treaty on medical matters," during the Ming dynasty.

Professional civet breeding for gastronomy has been practiced by certain Asian populations for several years, and is well developed in southern China and the province of Guangdong. The change of techniques for provisioning restaurants and markets, from hunting to organized breeding, already reveals in a few years of selection, that certain specimens are favored for their interest to the stockbreeder. The breeding at productive ends, involves physiological modifications characteristic of processes of domestication (increase in the annual number of parturition, increase in the weight, lowers aggressiveness) due to efforts of selection by the stockbreeder. These physiologic modifications which are made, are typical of the domestication process; faster production with reduced manpower, and is accompanied by an decrease of genetic diversity, which, associated with an increase in animal density, can support the incubation and diffusion of a virus and its variant virus in a breeding batch, thus increasing the probability of transmission to humans. It is in contact with these animals that stockbreeders and consumers contracted SARS [23,69]. In addition to clinical cases, several stockbreeders sero-converted without ever having developed SARS [70].

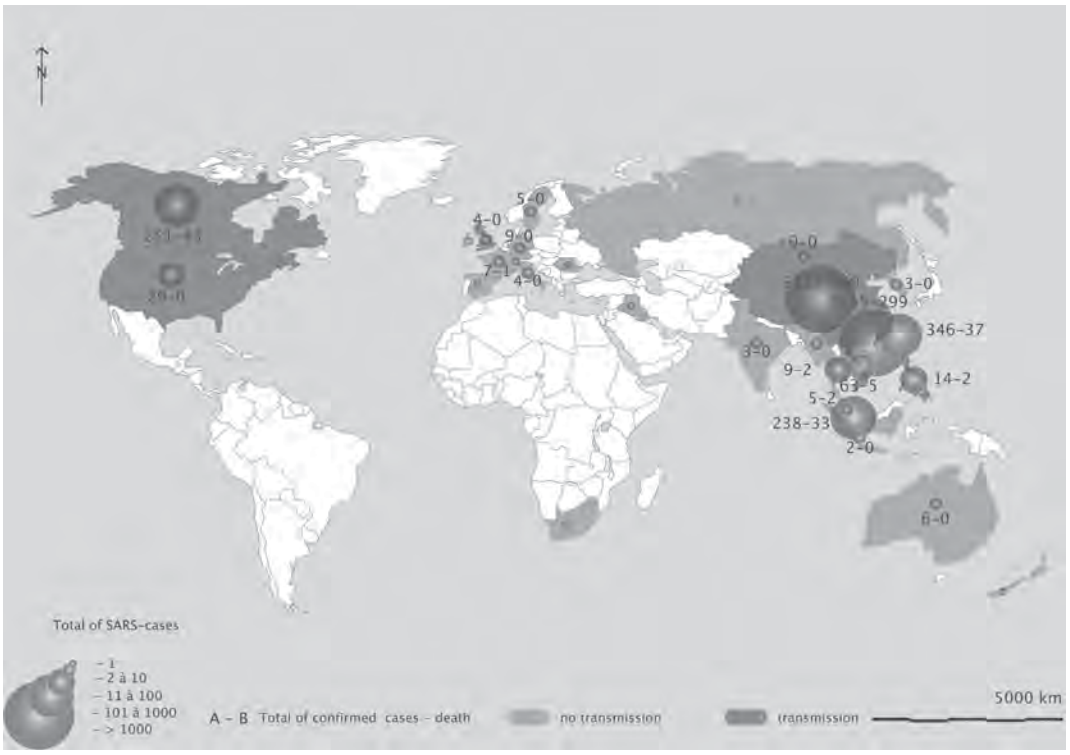

Fig. 32.22. Diffusion of the epidemic of SARS: accumulation of reported cases. (From data of OMS: http://www.who.int/csr/sars/country/table2004_04_21/en/index.html;http://www.who.int/csr/sars.)

32.3.6.2.3 CORONAVIRUS in livestock and [Sars-Cov] evolution potentiality The Coronavirus genus, included in the order of *Nidovirales* (linear, nonsegmented, positive sense, ssRNA viruses, comprising the families *Coronaviridae* and *Arteriviridae*) is well known to veterinary surgeons who observe it in many domestic mammals, in which it generally starts a serious enteric pathology, respiratory and/or neurological. Almost all domestic species and some semidomestic species are prone to infection with *Coronavirus*: pig (described in 1946), cow, turkey, chicken (1937), rabbit, dog, cat, and mice (1949) have all one or more *Coronavirus* which cause a high mortality during epidemics. Since the Sixties, a regular increase in the number of *Coronavirus* infections is observed. *Coronavirus* infecting humans with mostly enteric and respiratory tropism ([HCoVs], 229E and OC43) was discovered in the years 1960 [12].

The significant rate of evolution of the *Coronavirus* genus can be explained by three intrinsic factors. Coronaviruses are capable not only of recombinations in vitro but also in vivo (type) (case of Avian infectious bronchitis virus), they generally adapt without significant problems of deletions (1986 porcine epidemic of gastroenteritis due to a mutant) and finally, they do not have the mechanism of error correction of RNA, which is characteristic of the RNA-dependent RNA polymerases [28]. In the case of [SARS-CoV], a high rate of evolution was observed in a comparative study of evolution in [SARS-CoV] and in Nipah viruses of Chiroptera [43]. A large deletion involving 29 nucleotides (out of 30,000 in the genome of Coronavirus) occurred shortly after the passage of the virus to humans. This deletion, located between Orf11 and Orf12, which are not membrane proteins and which have unknown functions, does not seem to induce any notable effect.

32.3.6.2.4 An elusive virus reservoir: distribution and ethology of the host species and elements of the natural viral cycle In reference to the first isolation of [SARS-CoV] in wild animals and domesticated civets, these species were initially believed to have the role of the wild reservoir of the virus. But after the discovery of SARS-Like coronaviruses [SL-CoVs] in chiropteran, it seems more judicious to regard civet as semidomestic amplifier of [SL-CoVs] or intermediate host responsible of the transfer of the virus of wild origin to mankind. Multiple species of Chiroptera are carrying [SARS-CoV], among which, many bats, mainly insectivorous of the genus *Rhinolophus* (horseshoe bat): *R. pusillus* Temminck, 1834; *R. macrotis* Blyth 1844; *R. pearsoni*, Horsfield, 1851; *R. sinicus* [37]. Another species, *Rousettus leschenaulti* Desmarest, 1820, have sometimes anti- [SARS-CoV] antibodies. Two other species, *Miniopterus magnater* Samborn, 1931 and *Miniopterus pusillus* Dobson, 1878 can transport another coronaviruses [53].

The genetic variability of [SL-CoVs] is much more marked in chiropteran than in civet (and racoon dog) or humans, which tends to show on the one hand that the [SL-CoVs] from these species find most probably their wild

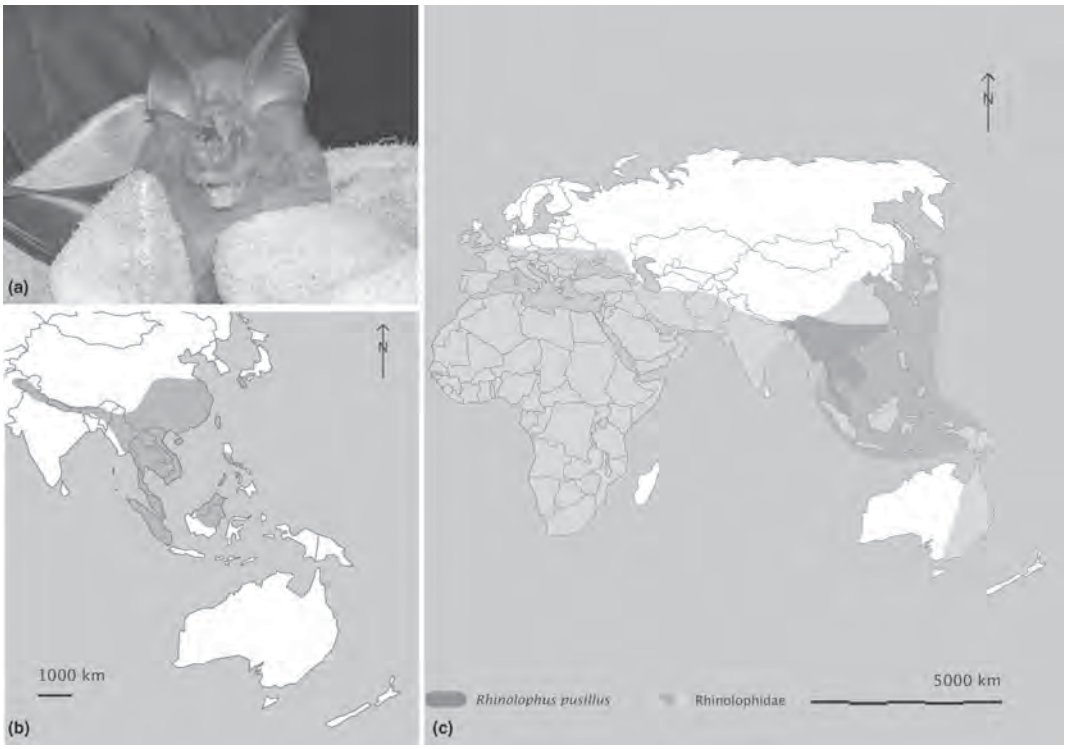

Fig. 32.23. (A) Chiroptera of genus *Rhinolophus*. (B) Range of *Paguma larvata*, the civet suspected to be the intermediate host of [SARS-CoV]. (C) Ranges of the family Rhinolophidae and of the species *Rinolophus pusillus*. The photograph shows the species *pusillus* Temminck, 1834 or *refulgens* Andersen, 1905 (caught in north–east of Lamphang, center–north of Thailand). From Refs. [11,40].

origin in bats, and also that Chiroptera have been hosts and carriers of [SL-CoVs] for a long time. In addition, as chiropteran show persistent infections by several groups of virus (Nipah, Hendra, Lyssavirus, Ebola, . . .) by developing very seldom a pathology, one can think of this group as a reservoir for certain coronaviruses and possibly for [SL-CoVs].

In the case of interspecific viral transmission, the question crucial is how it happens; either there is a great ubiquity of the viral agent conferred by a particular genetic material or a diversifying mechanism of extremely fast evolution and, able to produce viral communities with very variable potentialities. Coronaviruses have these characteristics.

The theory of quasi-species makes it possible to design mechanisms (as above) which ensure the perennially "of a virus": The virus remains and evolves thanks to the coexistence in the same host of several viral communities or populations, of the same origin, conferring on this viral agent a reticulated and diversified population structure, with probably the numerical and functional predominance of one of these quasi-species over the others. Consequently, a virus is not characterized by a monomolecular genome, but by a reticulated genome, composed of several variants, dispersed in a great number of viral particles, which have each one the possibility of repeating itself to give other lines, themselves more or less alternatives compared to the virus of origin. These various lines can also exchange genetic material in a horizontal way by recombination. In *Coronavirus* genus, this mechanism of recombination is frequent and give great evolutionary capacities.

In addition to these characteristics of coronaviruses, from the molecular level to the structure of viral populations, the characteristics of the host species are also determining in the evolutionary history of these viruses. The structure of these populations, the ethology and ecology of many species of Chiroptera carrying [SARS-CoV] are very favorable to the installation of viral mechanisms of evolution. The daily regrouping in large and dense colonies, the variable and sometimes weak periodicity of mobility (of the day or season range), the relatively heavy, mobile and diversified parasitic load, the mixture of species in sleeping areas and the long life expectancy (sometimes more than 20 years), are many factors supporting circulation, evolution, and diffusion of *Coronavirus* in general and [SL-CoVs] in particular.

Moreover, the various host species are generally distributed over large areas, partially covering each other, which allows for the meetings essential to interspecific passages of viruses (cf., Figs. 23B and 23C). For example, the *Rhinolophus* genus counts 69 species and is present in Australia and Europe. It is very probable that Chiroptera in general, and this genus in particular, still conceal several other coronaviruses, being given the potentialities of evolution and the ancient common history which the genetic diversity observed in these viruses suggests.

The settlements of Chiroptera are often of variable structure (changing specific diversity, effective unstable) and the populations widely fluctuate (variable sex-ratio, fluctuating structure of age) [36]. These observations suggest a strong mobility (daily or seasonal) of individuals and groups, within the range of distribution. Frequently, at the time of the operations of capture, one observes some individuals in bad physical condition (dull pelage, depilatory patches and open wounds on the abdomen or head, points of patagium necroses, charges heavy ectoparasitic burden). These individuals, weakened, but active since captured during their nycthemeral displacement, are more receptive to the viruses and may excrete them more and for a longer period than healthy individuals and thus can play the role of micro-reservoirs for local populations, dispersing the virus by their frequent movement.

32.3.6.2.5 Perpetual viral interactions with their hosts, a possible scenario for an emergence During emergence of new coronaviruses in the wild, by genetic drift, recombination or deletion, the genetic, ethologic and ecological characteristics of species of host determine the success of the process. Dispersion outside the species implies the existence of distribution surfaces overlapping those of other Chiroptera or other animal groups. In the case of [SL-CoVs], the passage in an intermediate animal host (civet) can be carried out by contact, licking, ingestion of fresh faeces from chiropteran or inhalation of dust contaminated in the caves (the ground of the caves is generally covered with a thick carpet of faeces dried and disaggregated into fine particles).

Civet, are preferentially nocturne like many small carnivorous, can be infected during night hunting in the caves and the trees where the bats excrete in great quantity from the middle of night to early morning. If the virus is well adapted to its new host, the infection is fast but this does not mean that it can be retransmitted as the individual may die or if the probability of meeting a congeneric is weak. In the case of a very virulent and very contagious agent, the individual will die and the virus will generally not be transmitted, except if the probabilities of meeting of a new host increase, for example as with the human activity of hunting (it is the case of Ebola, where humans are contaminated by another primate). In the case of civet, the original Coronavirus is undoubtedly not virulent enough to dangerously infect humans starting from only one meeting with a wild animal, but if the concentration of civets is increased artificially by breeding and the contacts human–animal are favored in the same way, the virus finds an excellent support for its evolutionary capacity and the emergence of alternative strains possibly pathogenic for humans is favored. Processes of domestication and breeding, by modifying ecological and biological characteristics of a species, and its relationship with humans, can then support the passage of emergent wild viruses from animals to humans. Afterward it is again humans who, by their behavior (sociality, manners) and the particular characteristics of modern life (fast global displacement, by airplane for example), disseminate the virus and contaminate other species and humans.

32.4 CONCLUDING REMARKS

The Emerging Infectious Diseases framework and the concept of a disease emergence both, as a system (together) have paved the way for developing preventive measures, controlling disease extension and forecasting epidemics at regional but also global levels.

The emergence of new diseases no longer has the exotic stamp, which stigmatized countries of the intertropical zone for being unhealthy territories producing plagues threatening to affect the occident. The concept of Ebola virus born form the darkest heart of Central Africa has matured, to the VIH suspected of a common geographical origin but this time quickly becoming pandemic, with the Avian flu virus, not yet pandemic but definitively pan-epizootic, or the Lyme Disease which, established slowly where its vectors (ticks) and their hosts (dears) broadened their field; and finally, with the chronic and degenerative diseases which, with another timing than the infectious diseases, emerge as much in the Northern countries (obesity) as in the urbanized areas of Southern countries (obesity, cardio vascular diseases).

Northern and Southern Countries, developing countries and industrialized countries all, know new diseases and have face the arrival of, up to that point, unknown diseases on previously virus free territories. However, if the emergence of new diseases is not the prerogative of countries known as "Southern," the populations of the developing countries are much more vulnerable than those of industrialized countries. This disparity is source of medical insecurity and increases inequality. To make cheaper curative treatments for developing countries is an evident task in modern medical thinking, which aims to protect vulnerable populations—to increase healthcare in the South for a globally better medical safety—and not to impose the same cost of curative treatments developed for a market of rich countries, in the context of tough commercial competition.

32.4.1 To Favor Prevention not Treatment

The emerging viral diseases are at the center of the debate on health in the developing countries of the often under medicalized intertropical zone where the epidemic manifestations have considerable impact on society and sanitary conditions. The priority of health for the developing countries is to prevent (fast diagnosis, systems of detection, control, protection) to avoid further treatment. It is this approach, which is necessary for the poorer populations. The double objective is to characterize emergence and, to identify and develop advanced warning systems for prevention of viral diseases.

32.4.2 The Emerging Viral Diseases Are Also a Growing Concern for the Northern Countries

A new Hantavirus in America; the encephalitic virus West Nile from Bucharest to New York, viruses of AIDS, a dramatic model of emergence, from germs in evolution to a pandemic of exceptional ampler and gravity. Lastly, beyond the nature there are emergent dangers of bio-terrorism and invented emergences.

32.4.3 Development and the Economy of Prevention

Emerging diseases and the emergence of the diseases have been and are of particular significance—and concern—when it comes to developing countries. Because the specificity of the phenomenon of emergence, including inedited germ or syndrome, a rapid extension of the disease, the unprepared population at risk, when such diseases strike it is over vulnerable population, with often a limited access to health care and limited medical support by all means. Consequently, the emergence of a disease in a developing country is multiplied by as much as the cited factors are deficient, the human and economical costs are of the same magnitude.

Moreover, many of the known emerging diseases in recent years, have their roots in the subtropical and tropical zones where most of the developing countries are situated.

Also, biodiversity exists as much for the microorganims and pathogens as for the macro-organisms, the tropical zone gives the richest biodiversity.

Altogether, developing countries are badly hit and also a cradle for numerous pathogens and other pathologies, which were known to be related to industrialized countries but became also a main health problem for developing countries facing rapid evolution of their societies in a changing world.

32.4.4 Diseases Will Emerge

New pathogens have threatened living beings since the origin of life on Earth. Unknown germs, new hosts and vectors will emerge as evolutionary process or in human knowledge. Growing human populations and global exchanges will intensively participate in the extension of new infectious diseases, as well as human and animal behavior, environmental changes of natural and anthropic origin will not only expose human populations to new germs and favor inedited epidemics to develop pandemics, but will also see the rise of chronic and degenerative diseases. Understanding the process of disease emergence will give the necessary tools and strategies in order that human beings continue to adapt in finding the right balance between control and prevention.

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CHAPTER 33

Epidemiology in a Changing World: The Need for a Bigger Picture!

J.-F. Guégan[1] and G. Constantin de Magny[1,2]

[1]*Génétique et Evolution des Maladies Infectieuses, UMR IRD-CNRS 2724, Montpellier, France*

[2]*University of Maryland Biotechnology Institute, College Park, Maryland, USA*

"... Quite properly, the medical epidemiologist's main concern is often with the recondite biological and medical details that make each infection unique ... In the absence of [such] a unified framework each infection tends to develop its own, often arcane, literature."

—Anderson and May, 1991

33.1 INTRODUCTION

Medical epidemiologists studying infectious diseases generally prefer to deal with their primary causes, propagation and prevention, rather than bothering with the possibility–when a communicable disease conforms to biological laws–of the existence of physical, ecological, or evolutionary processes that might affect the organisms in question. Epidemiology can be defined in a number of ways (there are many examples in this *Encyclopedia*), but traditionally it deals with epidemic manifestations, that is, the study of outbreaks of infectious diseases, their incidence and prevalence in human communities, and with establishing disease patterns and their aggravating factors. To use a simple comparison that should facilitate the task of the reader in understanding where we are headed in the present chapter, the study of infectious diseases until very recent times consisted only of examining the visible part of the iceberg, with the assumption that its nonvisible part was negligible and that the dynamics of the total ice mass did not interact with sea surface winds, marine currents and swells, or sea surface temperatures. This is a sensible attitude, because studying the interactions between, for example, disease spatial distribution and climate variability is already complicated, and necessitates collaboration with at least geographers and climatologists. Indeed, an enormous influx of nationally and internationally funded research resources have been targeted toward promoting sophisticated molecular tools such as genomics, proteomics, and nanotechnology (see many chapters in this volume). Clearly, this is of crucial importance in developing diagnostic tests, vaccines, and therapeutic tools, but what is really being studied is the health and diseases of individuals rather than populations and groups. Likewise, the traditional approach in medical epidemiology and parasitology has been to focus research attention on the understanding of local phenomena, considering that the unit of study is the individual, thus pinpointing the critical importance of sociocultural influences on disease patterns. We are not contesting here that socioeconomical parameters are not important in influencing the distribution and prevalence of disease, but we are arguing that some disease patterns can, in some cases, be attributed to the natural environment, as illustrated by the examples that will follow. Finally, there exists an individual-centered approach on the part of medical epidemiologists, which does not consider the effects of large-scale or global environmental influences; these

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can only be understood through the development of focused, comparative studies. However, the study of the health of human populations lacks a comparative health perspective, whereas major research developments have been made recently in other fields of life science, such as population dynamics and community ecology, that have benefited from such a comparative approach. Recent studies of the impact of global environmental changes on disease population dynamics and their spatial distribution [36,37,55,58] provide several good examples of how large-scale studies are of particular relevance to both wildlife and human epidemiology.

Several decades ago, scientists were still skeptical about the existence of a global environmental crisis; at present, however, they increasingly recognize the consequences of global changes in terrestrial, marine, and freshwater environments in the developed and developing worlds [2,3]. Evidence is also accumulating that global environmental changes may have a strong impact on the health of humans and even wildlife [15,20,34,36,60,77]. The issues involved are numerous [54,58] and highly complex; indeed, many different parameters acting at different spatial and timescales may be intimately interconnected in their effects [4], and these problems constitute a growing challenge not only for scientists and physicians but also for governments, international institutions, and societies [2,3].

Large-scale global environmental hazards to human and wildlife health include climatic change, ozone depletion, loss of biodiversity and ecosystem modifications, changes in hydrological systems and freshwater supplies, land degradation, and stresses occurring within food production systems [4].

In this chapter, we attempt an objective, though not exhaustive, analysis of global environmental changes and their impact upon disease patterns. We will concentrate on parasitic and infectious diseases, also referred to as communicable diseases, including emerging and reemerging diseases; we will not include noncommunicable and noninfectious diseases such as heart disease and hepatocellular carcinoma, for instance. Although primarily meant as a review, this chapter does contain some new information. The analysis of large-scale global environmental hazards in epidemiology requires integrating knowledge of different disciplines, thus necessitating a holistic research approach. The health consequences of global environmental changes are pervasive, and the different causes of the observed health patterns are often intimately interconnected. Clearly, problems of human health (and that of wildlife as well!) are complex, with many factors acting at different spatial and temporal scales. Our ultimate goal here is to convince readers, medical practitioners, research scientists, and even policy makers of the potential usefulness of enlarging the "window" of disciplines and subdisciplines so as to better understand and potentially control diseases. The implications are important for global public health issues, and this perspective should contribute to the design of public health programs in the very near future. As a title for this chapter, we chose to paraphrase that of a now-seminal contribution by Professor John Lawton [47], head of the Natural Environment Research Council (NERC) in the United Kingdom and one of the leading international ecologists, and to adapt it to the present exercise. As already stated above, major contributions have recently been made in macroecology and community ecology by adopting a comparative research approach; this is why our chapter is entitled "Epidemiology in a Changing World: the Need for a Bigger Picture!".

33.2 THE INTERACTIONS BETWEEN HUMAN POPULATIONS AND NATURAL SYSTEMS

Basic ideas and developments in medical epidemiology were developed in a world of "small, local communities," in which research priorities were given to case accounts of infectious diseases affecting human population groups, and their consequences for morbidity and mortality. With the appearance of large-scale global environmental hazards such as those we face today (some of which no doubt existed in the past, but which have been amplified by anthropogenic factors), the basic principles of medical epidemiology are being seriously questioned in light of their effect on local human health. This section is devoted to a brief description of why subjects in medical epidemiology are only rarely considered within a broad perspective, and why modern events such as global warming and other environmental changes due to a stronger human imprint on Earth should induce new ways of thinking in modern epidemiology. This section will end with an exploration of the different global environmental changes that may affect public health in the near future.

33.2.1 Human Psychology and Our Mental Perception of the Environment

Throughout the course of man's intellectual evolution, human societies have constantly been confronted with a range of problem-solving skills primarily based on local observations of natural phenomena. The first human societies were faced with the effects of lightning striking their houses or dreaded diseases; they tended to attribute such events to a local, perceptible mechanism, or else to an extraterrestrial, ideological phenomenon involving the intervention of a god or a spirit. The term "locus" is used in epistemology [35] to describe a scene in which human societies elaborate, in a preliminary state, the core of their societal organization: In other words, a clearing in the forest was the "locus" for Stone Age Celtic populations, and even now for native Amerindian tribal communities; populations were unaware of the role of potential external effects–outside the "locus"–upon their own living conditions. As human populations grew, they were faced with new events, and thus were obliged to take into account this external context for explaining local living conditions. The impacts of man-made global changes is now clearly recognized, as is the fact that such changes have yielded a vast array of problems, including those involving health. However, current trends in human transformation of the planet, including habitat destruction and climatic change, because they take place on

a large spatial scale, are not easily perceptible in modern human psychology. Our mental perception of the environment more efficiently copes with the directly proximal and perceptible causes. It must be recognized that the largest spatial scales–and this also is true for time scales–which cannot be easily evaluated from a locus-orientated perspective, may strongly influence lower hierarchical levels; this is indeed a prerequisite for modern science [57]. At the heart of any solution-oriented program is the need for training and convincing modern societies of the potential impact of global changes taking place at a lower level. In ancient Egypt, at the time of the Pharaohs, the wrath of God was evoked to explain major natural catastrophes. At present, a sudden explosion of locusts in Africa may have a lot to do with climatic variability at a continental or global level.

33.2.2 A Changing World, Changing Human Mentalities, and the Role of Science

Conceptually, much of our mental perception of the environment takes root in the direct connections we observe between cause and effect on a very fine spatial scale. However, the consequences of global changes constitute a reality that we can no longer ignore; we need to readjust our thinking when confronted with complex environmental problems. As already stated, the issues are numerous, of great complexity and often interrelated [1,87].

Human health systems are complex entities characterized by the diversity of their components, dynamic interactions within and between these components, and their relationship with the environment. None of the standard entities usually employed in epidemiology, for example, genes, individuals, communities, are completely separate from the others, and any one of them is embedded in a continuous hierarchy of structural organization (Fig. 33.1). Most epidemiologists would agree that it is not an easy task to use observations at a small, individual level to predict what will happen within a larger ecosystem, or, *vice versa*, or to determine which macro-level process is responsible for micro-level patterns. For instance, in epidemiology, the many problems we face, for example, vaccination and herd immunity, or the role of climate change in disease dynamics, cannot be solved without properly addressing the complexity of host–pathogen interactions, including their global environmental and human dimensions. Modern epidemiology is now confronted with the problem of how to identify the relevant spatiotemporal and organizational scales that might be relevant in explaining disease patterns and processes. A new integrative approach and a theory of up and down environmental scaling, involving both abiotic and biotic phenomena, is needed in epidemiology [33]. Epidemiology today must deal with problems associated with linking processes on very different spatial, temporal, and organizational scales (Fig. 33.1), and with health-related inducers of global environmental change probably representing the forgotten dimension in global health [1,4,33].

One major problem faced by human societies, and by the host planet Earth itself, is that of increasing population

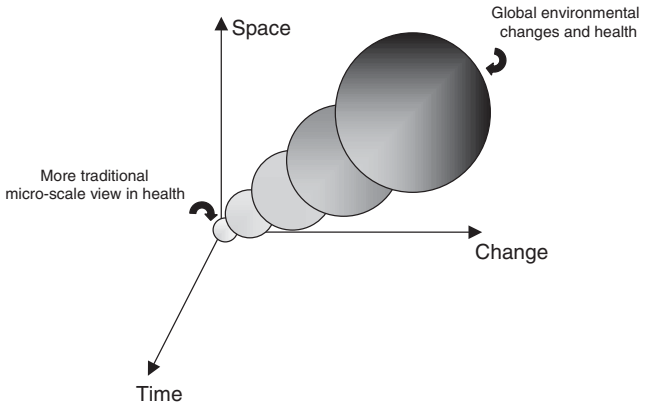

Fig. 33.1. Human (and wildlife) health concerns intervene on a three-dimensional scale, that is, space, time, and change. Because of this hierarchical organization, in any health system, scaling laws must be taken into account for health research and public health strategies.

numbers and expanding human footprints exerted upon the planet. Humanity as a whole has seriously exploded in size, meaning that human consumption requirements will not be fulfilled by the resource capacities of the planet in the very near future. In other words, we are at present in a state of rupture in the global production-ecological capacity of the Earth's equilibrium [1,95]. At the heart of any global health planning policy is the consideration that humanity cannot increase in size as it is currently doing because the global support capacity must not be exceeded. But, how did we arrive at this critical point?

In earlier times, human populations–and this is also true for certain, now rare populations on remote islands and native tribes in the rain forests of Amazonia, Papouasia-New Guinea, and Central Africa–lived in harmony with their natural ecosystem, which was able to meet the community needs (Fig. 33.2a). As human populations exponentially grew in modern times with the appearance of community settlement encroachments in new areas, the impact exerted by human societies on ecosystems (Fig. 33.2b) became greater and greater. Human ecological footprints on the planet, defined as the extent to which human consumption can be supported by local ecological capacity [1,95], has grown in such a manner that there is no comparison in time. Deforestation, habitat fragmentation, land use, and agricultural systems, coastal zone and watershed management, climate change, ecotourism, transportation trade and transcontinental traffic, added to new behavioral practices, have created new conditions for more direct contact between humans, ecological communities and ecosystems (Fig. 33.2c). The emergence of new viruses and bacteria, as recently observed in some human communities [60], is the immediate manifestation of the fact that human and animal disease interactions have become more frequent and human societies are strongly impacting on the ecosystem equilibrium (Fig. 33.2c). The ecological effects exerted by humans may have dramatic consequences that extend throughout a cascade of ecological

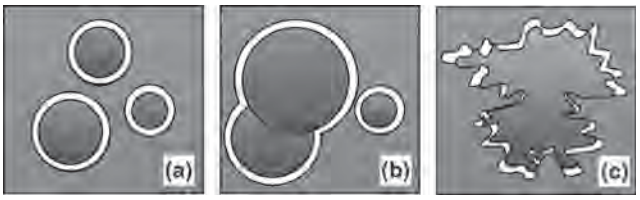

Fig. 33.2. Schematic illustration representing the relationships between human populations and their environment. Grey forms indicate human communities, grey squares natural habitats such as rainforests, and white forms the contact margins between the two domains: (a) ancient human populations, and even some present-day native populations or isolated populations on remote islands, living in harmony with their natural habitats; (b) with the population explosion and new community settlements, the interactions between humans and ecosystems have increased, creating more and more contact areas between the two domains; (c) current situation in which human populations strongly increase in size and natural ecosystems largely interact, as the human footprint on the planet expands.

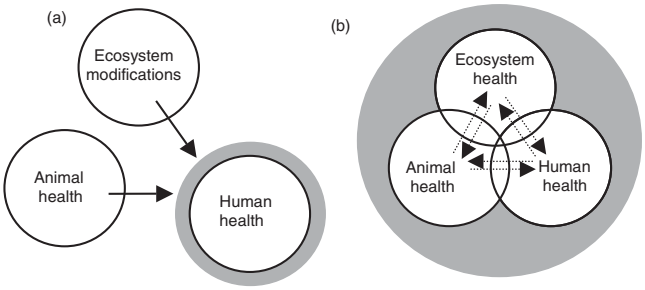

Fig. 33.3. Schematic diagram illustrating: (a) the traditional view, in epidemiology, of the links between human health, ecosystem modifications and animal health; human health problems are seen in a downstream perspective where only the impacts of ecosystem modifications on human health are considered (see arrows); (b) conceptual diagram of possible multiple two-way links between human health, animal health and ecosystem health, in which all the components interact. Conservation medicine as an interdisciplinary field addresses the complex interrelationships between health and ecological concerns. Modified from Ref. [1].

responses. Clearly, these problems raise questions about the scientific and social attitudes required in order to meet the challenge created by such complex issues.

Thus, traditional health concerns have been oriented toward the downstream effects of environmental impact [87], such as those of pollutants or toxic substances upon human health, or habitat modifications upon disease dispersion, notably in occidental industrialized countries. This cultural habit of examining downstream events in medical epidemiology instead of upstream events, for example, understanding the proximate and ultimate causes of disease (re)emergence, needs to be reinterpreted (Fig. 33.3). The true impact exerted by global environmental changes on health should compel medical scientists to rethink several basic assumptions, and notably that of the "bottom-up" approach as a single research perspective in the search for more general proximate causes for observed morbidity and mortality in local communities.

Looking to the future, a key goal is to move from a consequence-oriented method to an "ecological-context-of-health" framework (Fig. 33.3), in which we would take into consideration the multiple two-way interactions between pathogens and disease, and between the many host, vector, reservoir, and microbe species that may be involved within ecosystems [32].

This requires a better integration of knowledge of different disciplines and subdisciplines in order to elucidate complex relationships between health and the environment. This intellectual trend is referred to as *Conservation Medicine* [1]. A move in this direction clearly requires an interaction on the part of medical science and related disciplines. The plethora of findings described below clearly suggest the need for extending health questions to other disciplines such as climatology, oceanography, physics and mathematics, population biology and genetics, or community ecology, for instance.

Finally, as already discussed in previous sections, the many problems we are facing today, for example, climate change and disease dynamics (see examples hereafter), cannot be solved without properly addressing the complexity of host–pathogens interactions, including the appropriate scaling law level of resolution at which specific processes may happen. Interestingly, recent studies on the impacts of global environmental changes on disease population dynamics and their spatial distribution [20,34,60,77] have provided good examples of how large-scale studies are of particular relevance in coping with human health problems. In order to deal with large-scale patterns and processes, comparisons between data and model outputs are required in order that a "bigger picture," to use the terminology of Lawton [47], may emerge. Although major research developments have come about recently in other fields of life sciences such as population dynamics, community ecology, and macroecology [7,57,80], largely due to the use of comparative research perspectives, epidemiology continues to suffer from the absence of comparative studies, because of the individually focused-research dimension given to health problems. We must go beyond local details upon which traditional health investigations have usually focused, and search for more general and rigorous disease patterns [6,79], thus providing a consistent framework upon which to build a true predictive discipline (see Fig. 33.4).

As recent understanding of some infectious disease patterns such as environmentally persistent, zoonotic and vector-borne diseases, have clearly shown the impact of large-scale climate variability on the geographic spread of these infections [39,60,77,78], medical epidemiology clearly needs to go one step further in developing comparisons between local data (Fig. 33.4). Recent investigations into childhood diseases have also provided clear evidence of how large-scale studies on a nationwide or global scale are of particular relevance for public health concerns in the characterization of the many processes involved in disease behavior (from Refs. [6,79], see also Chapter 12 in this volume).

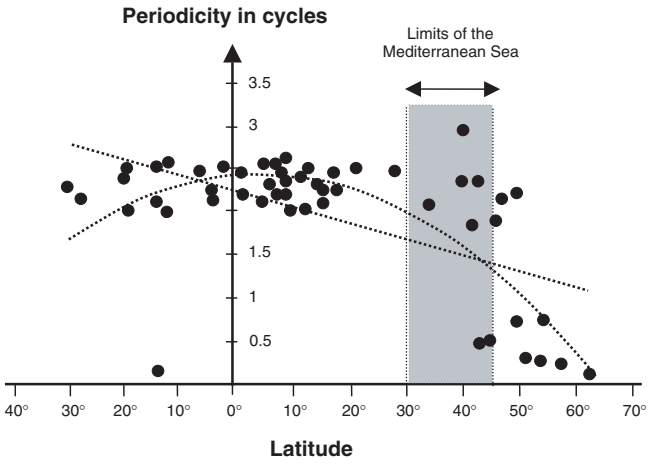

Fig. 33.4. The comparative approach based on comparison of independent data sets at lower scale, for example, time series for cholera at a nationwide scale, is a way to characterize disease patterning at larger scale. Data show the evolution of cholera population time series dynamics in terms of shape of dynamics, that is, highly regular and periodic to erratic. Dots synthesize the form of the disease population dynamics on a double-axis gradient: On the *x* axis is the position in latitude, at which the disease cases come from; on the *y* axis is an index value with estimates near 2 representative of cyclic population dynamics of cholera, and near 0 of highly erratic dynamics. The figure shows that the evolution of cholera population dynamics from southern Africa to northern Europe shows cyclic fluctuations in tropical and subtropical areas of Africa, and when moving toward southern and northern Europe disease cases are rare to extremely rare. Population dynamics of cholera cases is transient in the Mediterranean basin (see grey rectangle), with epidemics of cholera more or less regular on the seashores of northern African countries (data from Guégan and Constantin de Magny). Another illustration of the comparative approach in epidemiology is given by Broutin et al. [6]).

From traditional investigations focusing on very small-scale case studies in medicine, there is a growing scientific tendency, under the impetus of population biologists, to go one step beyond the idiosyncratic details at a lower local scale and to move to a broader perspective of epidemiological systems in order that only the important disease generalities or patterns remain [6]. Because medical epidemiologists primarily focused their research investigations on local qualitative details in explaining disease patterns, they did not consider, or else totally neglected, the importance of large-scale processes, arguing that comparison across localities was impossible due to data heterogeneities. On the contrary, the main focus of comparative analysis is to compare data acquired at a lower spatial scale–and thus not to decide *a priori* that comparison is totally impossible–and to consider that emerging patterns may exist at a larger scale encompassing the total dataset under study. The basic idea in comparative analysis in epidemiology is to describe the different spatiotemporal patterns that may be at work at the different hierarchical scales under scrutiny and then to explore the corresponding processes responsible for the observed patterns. Comparative analysis is thus a promising approach to public health concerns in infectious disease population dynamics, in that it offers a much broader perspective on health and a more quantitative approach to predicting and controlling disease evolution.

33.2.3 Global Environmental Changes: New Health Threats for the Foreseeable Future

Medical doctors, researchers, policy makers, and the general public are relatively unfamiliar with the nature of global environmental changes and with the very complex links that may exist between these global changes and human well-being and health.

Global environmental changes refer to planetary-scale, largely human-induced alterations that affect the environmental capital of the planet to support life, and which modify the structure, composition, or function of large-scale biophysical and ecological systems [1,55,58]. They are by-products functions of an unprecedented situation in which the overall population size of the planet is dramatically increasing at an unprecedented rate, and in which economic activities and technological choices are beginning to considerably modify aspects of the planet (see above). Global human-induced environmental changes may be either those environmental changes that impact on global process, for example, the aggregation of greenhouse gases, which amplify the worldwide greenhouse effect, or widespread local changes, for example, ecosystem modifications or desertification, which by accumulation on a large scale, may modify ecosystem functioning. What is common between all these human-induced environmental changes is their "global" dimension and the uncertainty as to their possible consequences for human societies, as their existence is new. They differ as such from localized toxicological and microbiological environmental hazards, the consequences of which never cross the upper scales.

Global climate change is undoubtedly the most well-known pattern of global environmental change, but other types of global changes exist that pose serious problems for the future of our planet's life. The main types of global environmental changes induced by humans are (i) changes in the atmospheric composition with stratospheric ozone depletion and greenhouse gas accumulation, (ii) biodiversity loss and changes (e.g., biological invasion and extinction), (iii) disruption of elemental cycles, for example, nitrogen, sulfur, and phosphorus, (iv) changes in the hydrological cycles and depletion of freshwater supplies, (v) changes in food-producing ecosystems (e.g., land cover, soil fertility, coastal, and marine ecosystem stocks), (vi) global dissemination of persistent organic pollutants, (vii), urbanization, and (viii) desertification [1,55,58,87].

As we have already stated, the health of human communities is strongly influenced by large-scale conditions from beyond the boundaries of those communities' living space, that is, the locus. Consequences of global environmental changes on health may be complex because many different effects may interact with each other and with lower scale changes that make their understanding and prediction highly

difficult. Although more and more researchers are currently working on global human-induced changes in health, this domain is quite new. Modern-day environmental threats and the risks they may induce in human societies should thus stimulate more research initiatives so as to provide new skills, new tools, and new vision of global health.

33.3 DYNAMIC PROPERTIES OF MICROBES, THEIR HOSTS AND THE ENVIRONMENT

There exist many diseases and disorders that are environment-dependent; we can here cite the heat-wave-related mortality that occurred in France in the Summer of 2003, or the numerous parasitic and infectious diseases that have increased, or will increase, in prevalence, incidence, and geographical range distribution. In this chapter, we will focus only on communicable diseases, but the readers may refer to [4,52,55,58] for further details on noncommunicable diseases. They will also find more information on the effects of global environmental changes upon health and human well-being at the World Health Organization website at http://www.who.int/globalchange/en/.

But, how can global environmental changes affect human health? Even though the connection between natural history and medicine is generally accepted–at least in recent textbooks–this paragraph is devoted to a general presentation of the linkages that really exist between ecological processes on Earth and health. We will begin this section by describing the broad ecological context of infectious diseases. Then, we will explore the many two-way interactions that truly exist between ecological processes, microorganisms, and health. We will conclude this section by discussing the emerging field of *Conservation Medicine*, which brings together environmental sciences and the many disciplines of health.

33.3.1 The Ecological Context of Infectious Diseases: The Three-Piece Puzzle

Although much has been learned about parasitic and infectious diseases, their potential natural hosts, reservoirs and vectors, and their interactions with the environment, many questions remain. An ecological perspective in health science will reveal that we are still ignorant of the complexity of interactions such as those of food chains, and the diversity of microorganisms that truly exist on Earth, some of them being potential human pathogens [32,91].

A recent outbreak of a very rare zoonosis attributed to the monkeypox virus (MPV) in the Spring of 2003 in the central United States, which caused an illness clinically indistinguishable from smallpox, made the headlines [16]. Sporadic cases of monkeypox virus had been previously reported in human individuals only from the rainforest areas of central and western Africa, where the main reservoirs (throughout primary origin remains unknown) are squirrels (*Funisciurus* and *Heliosciurus* genera), Gambian giant rats (*Cricetomys* genus), and certain species of monkeys. This outbreak had never been recorded in the northern hemisphere before and was occasioned by the international shipment of small African mammals from Ghana to Texas, thereby constituting the source of disease introduction. From the six different categories of rodents introduced in the United States for pet distribution, at least one Gambian giant rat, two squirrels, and three dormices (*Graphiurus* genus) were identified by medical authorities to be infected by monkeypoxvirus. Native prairie dogs (*Cynomus* genus) co-housed with Gambian giant rats in pet shops were contaminated. Fortunately, none of the human cases identified in the United States resulted in death, and most of the patients did not fall seriously ill [16]. The establishment of a new zoonotic disease in the United States was avoided. Because its initial clinical features are indistinguishable from those of smallpox, a dreadful mutilating, and even killer agent for humans before its eradication in the 1970s, lessons were learned from the latter. Recently, Smith and collaborators, using an impressive dataset of information on human infectious diseases, have demonstrated that disease categories that are limited by the export of their host species between nations and continents, that is, multi-reservoir and zoonotic infectious diseases, are most likely to emerge in entirely new regions of the world [83]. This is particularly alarming in the face of increased rates of exotic species introductions, notably in developed countries (see Section 33.4.6).

The relationships between microorganisms or parasites, their hosts and the environmental conditions, both physical and biological, under which all these components interact, have developed throughout a long history of community coevolution [97]. As explained by community ecology and population dynamics, local animal communities–and within them the important components known as microorganisms–are constantly exchanging fluxes of energy and materials with the surroundings [32,71,92]. Local communities, and thus pathogens within them, are ultimately dependent upon a balance between the rates of migration and extinction throughout ecological times. This necessitates a consideration of balance in nature as a starting point of discussion on infectious disease dynamics in space and time (Fig. 33.5).

As illustrated in Figure 33.5, the conventional statical view of health is now replaced by a more complex, dynamic three-piece puzzle in which the component parts, that is, the host, the agent and the environment, abiotic or biotic, strongly interact with each other and where multiway interactions, feedbacks and loops may intervene in disease behavior in time and space. As such infectious diseases cannot be viewed as a separate, independent entity apart from the whole ecosystem–the conventional downstream approach to health–but rather, need to be considered as a piece of a more complicated puzzle in which all components are extensively interdependent on each other; in other words, any effect on one subset may potentially lead to consequences for the others [97]. Because of the reality of this diversity of interactions and other linkages between the agent, the host and the environment, health problems, at least when it concerns communicable diseases, have much to do with complexity, rather than

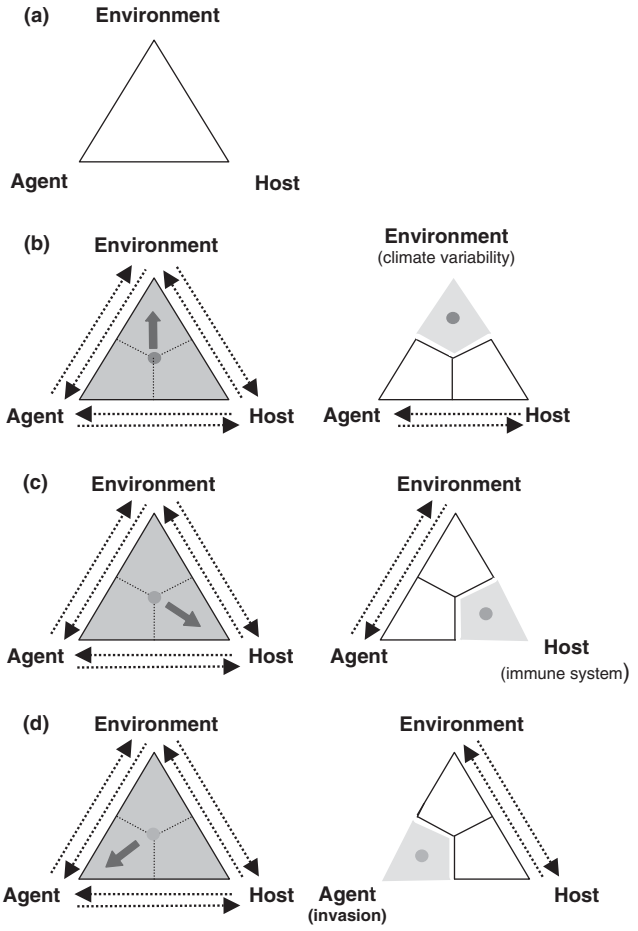

Fig. 33.5. Schematic representation of the infectious disease triad of agent, host, and the environment: (a) classic, static representation of the three-pieces triangle; (b)–(d) dynamic illustrations of the three-pieces triangle which considers two-way multiple interactions between components of the triad (on the left); dots illustrate a given disease, and the arrows the major force explaining disease patterns. On the right side of the figure: (b) case where the environment, for example, climate change, is important for explaining disease patterns such as for many vector-borne diseases; (c) case where host biological conditions, for example, immune properties, are preponderant, for example, HIV spread; (d) case where intrinsic factors of the etiological agent are important in disease dispersion such as for influenza virus. Modified from Ref. [97].

being simple cause-and-effect relationships. One pertinent example of such complexity is the demonstration of how the interplay between environmental forces and ecological responses may mould the population dynamics of disease. A cogent illustration is that of the cholera epidemic resurgence in certain populations, which clearly shows the existence of cyclic fluctuations in disease case numbers (Fig. 33.6).

One important question is whether cholera outbreaks in human populations are simply the acquisition, by specific bacteria strains, of virulence genes, that is, genes for the phage-encoded cholera toxin and the toxin-regulated pilus factor [21], responsible for better transmission dynamics,

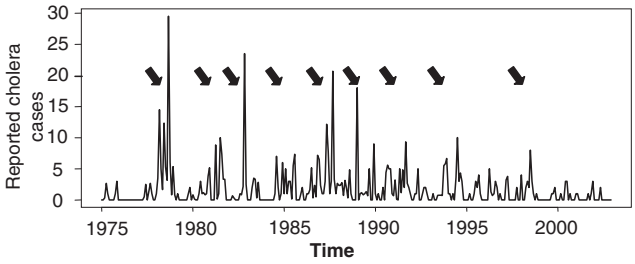

Fig. 33.6. Evolution of the number of reported cholera cases (monthly data) declared to the World Health Organization by national health authorities of Singapore as a function of time (1975–2002). Cholera population dynamics shows somewhat seasonal patterns of epidemic resurgences and interannual largest epidemics each 5–7 years (see black arrows). From de Magny and Guégan' compiled data.

which clearly represents a fine-scale, direct cause-and-effect approach on health, or whether they are due to a complexity of factors involved at different time and spatial scales that determine disease reemergence, which in fact constitutes a holistic perspective on health [11]. Studying disease from the unique small-scale molecular perspective seems incomplete, because it does not totally explain why recurrent epidemic waves occur in time (but see Ref. [22]).

First, the variation in fraction of susceptible individuals in the population over time as the result of immunity acquired by previous infection, and by the input of births and migrants into the pool of susceptible hosts, may result in that the population dynamics of cholera fever intrinsically oscillates and creates annual to biennial epidemic outbreaks. But, in addition, when strong couplings between climate and transmission occur like during *El Niño* events, a second outbreak is then observed each 5–7 years like in Bangladesh [67]. There is considerable evidence by now linking cholera outbreaks to climate [11,68,69,84], yet mechanisms explaining this coupling are not well understood [67]. Temperature, rainfall, and plankton blooms among others have been proposed to explain the seasonal nature of cholera. Magny and collaborators have recently provided a mathematical model for cholera population dynamics in human communities that incorporates the seasonal dynamics of phytoplankton blooms–a potential reservoir for bacteria–and *Vibrio* concentration in aquatic environments [53]. The readers will find an excellent report from a scientific committee by the US National Academy of Science on the impacts of climate and weather on infectious disease at http://www.nap.edu/catalog/10025.html.

Obviously, other environmental parameters are important in the emergence, incidence, and intensity of cholera outbreaks. Studies have shown that avirulent environmental strains of the *Vibrio cholerae* bacteria are maintained within the ecosystem, and may be associated with phyto- and zooplankton organisms, algae, crustaceans, and fish [12,41–44,56,82,90]. Thus, the ecology and transmission

biology of *Vibrio* forms are twofold: (i) probably many environmental avirulent forms live in natural ecosystems, but there is limited understanding of their exact life cycle; and (ii) certain particularly virulent strains are selected to invade human populations. The second phase, that is, transmission from person-to-person, which in fact represents an evolutionary "dead end" for the bacteria, is familiar to the public, and it is generally that which is studied by medical epidemiologists, and it forms what we have called the emerging part of a larger iceberg. In reality, what is currently poorly understood, that is, the environmental phase, should in a very near future prove to be the cornerstone for a better understanding of bacterial evolution, for example, genetic polymorphism, gene transfer, and strain adaptation to specific contexts. Recent findings [22,23] have clearly shown that bacterial viruses in both the environment and the intestine of cholera patients might strongly influence cholera cases seasonality in that *in vivo* bacteriophage amplification in infected individuals and hence bacteriophage predation on environmental *V. cholerae* during the cholera epidemic yielded the collapse of the disease. These data suggest that bacteriophages in the aquatic environment are an additional factor that causes disease population dynamics in human. However, it seems also likely that specific climatic conditions may lead to the explosion of bacteriophages in aquatic environments that may cause changes in the abundance of *V. cholerae* bacteria, a phenomenon recently observed for gastrointestinal nematodes of red grouses [8]. The incidence of cholera is also affected by many other factors like sanitation and level of poverty, public health services, population demography, land use changes and urbanization, and travel exchanges, for example, the pilgrimage to Mecca, and these are commonly invoked to explain cholera outbreaks and dissemination within populations (see Chapter 37 in this volume). Thus, the assembly of the different extrinsic and intrinsic factors into a perspective–in a multiple pathway diagram with both direct and indirect connections between the many parameters involved on different spatiotemporal scales–should be a prerequisite for better capturing the complex web of causation that shapes cholera epidemic resurgence over time. This leads us directly to the next section on the linkages between ecosystem dynamics and infectious diseases.

33.3.2 Ecosystem Dynamics and Health, or the Snowball Syndrome

Any new outbreak in a population usually generates much public and media speculation on the possible causes underlying such phenomena. The avian flu outbreak in Asia between late 2003 and the beginning of 2004 is informative in that wild birds, particularly waterbirds, have been blamed for the serious spread of the disease in Asia, whereas poultry ducks are considered to have played a central role in the generation and maintenance of the flu virus [49]. Policy authorities in Thailand even got to the point of ordering a cull of openbill storks, a long-distance migratory bird species, although this decision was then called off. Confronted with panic, the Food and Agriculture Organization (FAO), the animal health service of the United Nations, urged Asian public authorities not to cull wild birds, because there was no direct evidence that they were responsible for disease spread between the different outbreaks, and "wild birds are an important element of the ecosystem and should not be destroyed" (see FAO news at http://www.fao.org/newsroom/en/news/2004/48287/index.html). Although it is entirely natural to search for the more plausible factors involved in these issues, it should be emphasized that a complex web of causative factors with multiple interactions and environmental forcing may intervene, and thus the political decision making openbill storks the scapegoats was unwarranted!

The relationships between ecosystem dynamics and infectious disease are obviously, highly complex in the patterns and processes involved, and totally underestimated or poorly understood because of the lack of research studies that could help to disentangle this natural biological complexity [15]. In addition, the links between ecosystems, communities, and health are often strongly dependent on local factors, for example, local conditions, which predispose the spread of an emerging zoonotic disease to human communities, whereas these links are more vague on larger spatial scales, for example, the aggregation of deforestation surface areas, which might amplify the risk of new infectious diseases emergence–but all this needs to be formally demonstrated. Changing ecosystems, that is, due to modifications, instabilities or perturbations, induced or not by climate change, land use changes and stress, loss of biodiversity, species dislocation and alien invasion, may have strong connections with public health problems in that any effect on the planet's ecosystems could have repercussions, in a cascade-and-effect scenario, upon human beings [9]. This is what we call here the "snowball" syndrome, in which tiny local effects may produce bigger problems on a larger scale.

One of the best-documented illustrations on the link between ecosystem dynamics, community, and health issues is Lyme disease [65,66]. Lyme disease is a tick-borne disease caused by the spirochete bacteria, *Borrelia burgdorferi*, and it most often presents with a "bull's-eye" rash and erythema, accompanied by nonspecific symptoms such as fever, malaise, fatigue, headache, muscle and joint aches. Lyme disease is considered to be an emerging disease in western countries. In the United States, where the spatial dynamics of Lyme disease has been investigated [65,66], it was shown that the risk of disease transmission strongly depends on local vertebrate-species diversity. In habitat patches with high vertebrate-species richness, many vertebrate species may be bitten by infected ticks (from the *Ixodes* scapularis species), feeding from a wide variety of mammalian, avian, and even reptilian host species. However, all these potential hosts strongly differ in their probability of infecting a feeding larval tick. In eastern North America, the white-footed mouse is highly efficient at infecting feeding mites, but other species are poorly capable of, or totally incompetent at, transmitting the bacteria to feeding tick larvae, thus impeding or interrupting the disease life cycle [66]. High-order vertebrate-species diversity effectively

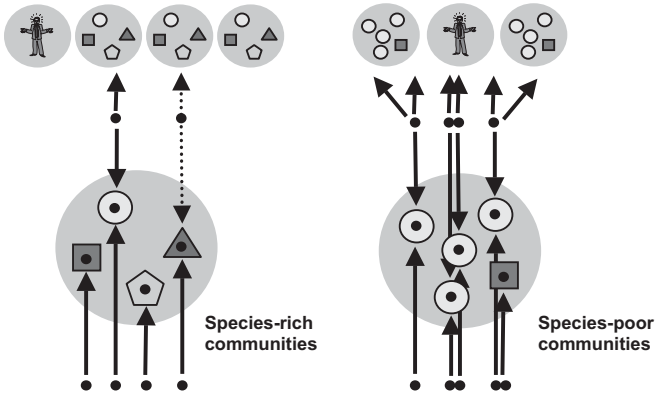

Fig. 33.7. Schematic representation of the ecology and epidemiology of transmission of Lyme vector-borne disease. On the left, in local species-rich communities of vertebrate hosts, potential host reservoirs do not transmit the bacterial agent with the same probability. Some host species (green disk) are more efficient at transmitting the disease to biting ticks, whereas others are poorly able to do so (red triangle and dashed arrows) or unable to do so (blue square and yellow hexagon). On the right, in local species-poor communities, where a more efficient host reservoir occurs (green disks) the disease is transmitted at a higher rate to feeding tick larvae, and thus the probability of human contamination is higher. Ostfeld and Keesing [65,66] have called this ecological phenomenon a "buffering effect" conferred by biological diversity, which tends to dilute the disease agent. See color plates.

locally dilutes the disease agent, and risk of disease transmission to humans is thus less likely (see Fig. 33.7).

Richard S. Ostfeld and Felicia Keesing [65,66] have referred to the potentiality that biological diversity may exert on infectious disease transmission as a "buffering effect" conferred by effectively diluting large fluxes of disease germs into poor or nonefficient hosts vertebrates. A by-product of this within-local-community disease agent dilution is that it is less likely, in terms of probability, for an infected tick to transmit the Lyme disease to humans (Fig. 33.7). On the contrary, when local communities are species-poor and a more efficient host reservoir comes into being, larger fluxes of disease agents are generated, which increases the risk of disease contamination to humans (Fig. 33.7). Another complementary but not exclusive explanation proposed by these authors is that competitors and/or predators of the main Lyme disease reservoir keep local mice populations low, thus reducing the risk of disease transmission. Ostfeld and Keesing [65,66] have opened up a new and fascinating avenue of research on the role exerted by biological diversity, community composition, and wealth on infectious disease ecology and epidemiology. Based on basic knowledge, we thus suspect that many other infectious diseases (around 63% of current human infectious diseases are zoonotic), and animal diseases as well, might be driven to some extent by the same ecosystem dynamics rules, suggesting that practitioners, policy makers, and the public should more carefully evaluate interconnections between ecosystem, biodiversity and health concerns in coming years [15,48,72,73,88].

Humanity is degrading Earth's ecosystems at an incomparable rate, disrupting their functioning while human populations continue to live at the expense of a number of ecosystem services: nutrient recycling on land and in the oceans, detoxification of many dangerous substances, air purification, fossil energy production, or food production [48,72,73,88]. Both ecosystem functioning and biological diversity are cornerstones of the Earth's magnificent architecture, and it is up to humans to find their exact place in this system.

33.3.3 The Emergence of Conservation Medicine

The many aspects of research on ecosystem health and human health discussed above, in which our safety and wealth strongly depend on the ecosystem and on community health as a whole have helped to define a current of thinking now known as "*Conservation Medicine*" (see website at http://www.conservationmedicine.com/), the main goals of which are (i) a better understanding on the many inextricable interrelationships that may exist between human health, animal health and ecosystem functioning and dynamics; and (ii) the promotion of such thinking in different arenas such as those of policymakers, economists and the public [1]. The many examples that we have chosen to show below are representative of this promising field. The reader will also find more details on *Conservation Medicine* in excellent books by Aguirre and collaborators [1] and Lebel [48], (see also http://www.idrc.ca/ecohealth). A free online French version of Lebel's book is available at http://web.idrc.ca/en/ev-32399-201-1-DO_TOPIC.html. Previous important publications by the *Consortium of Conservation Medicine* include contributions to two books on conservation biology [88] and world sustainability [72], respectively.

33.4 THE ECOLOGY OF INFECTIOUS DISEASES IN PRACTICE

Although medical epidemiology has focused on the socioeconomic determinants of disease patterns, we still have a very limited quantitative picture of the geographic distribution of the main disease agents in human populations throughout the Earth [45]. To what extent are they climate-dependent, and how many of them are there? What are the quantitative linkages between organism biodiversity and human pathogens? What are the main drivers of disease emergence or reemergence and dispersion, excluding the generally invoked socioeconomical drivers? Are socioeconomy and modernization really important in explaining disease spread, and for which diseases are they pertinent? Recently, McMichael [59] has provided some examples of emerging infectious diseases considered under major categories of environmental and socioeconomical influences, and we recommend the reader to refer to this contribution. The following sections deal with how to disentangle the complexity of the many interrelationships that truly exist between ecosystem

functioning and its impacts on health. Examples may come from the general published literature or they may be new illustrations from personal findings by the authors of this chapter.

33.4.1 What Came First: Biology or Socioeconomy?

As the roots of current epidemiology are strongly embedded in the socioeconomic development of western countries [45], what we call "mimetic epidemiology," epidemiologists have primarily focused their research efforts on the importance of modern conditions, for example, sanitation, urbanization, and economic activities, in infectious disease patterns. Two questions tend to dominate the traditional approach by the epidemiologist to disease study: where? And, why there? Determining the "where" has led to considerable work in observing, identifying, and depicting qualitative patterns of disease spatial distribution. Understanding the "why" focuses on the importance of social and technological organization and rapid environmental changes–the so-called economically based approach –as major processes influencing infectious disease distribution and occurrence. Very few studies have attempted to study in a thorough manner the spatial distribution of the many pathogen species in humans as a group, and to analyze the factors affecting their geographical ranges. The most important developments in modern epidemiology involve the search for epidemiological–ecological patterns, regularities and order in space, and then explaining these observed patterns by the many abiotic and biotic processes, which are interactive and which operate differentially in space and time, generating, modifying, replacing, and eventually destroying such patterns.

Recent statistical insights into the spatial distribution of infectious and parasitic diseases in humans on a large scale have enabled robust predictions of the different mechanisms responsible for the observed patterns. Using extensive datasets on up to 332 different human pathogens throughout the world, Vanina Guernier and co-workers [34] have shown that, after correcting for covariates, they still observe that species richness in human pathogens is strongly correlated with latitude and, in general, human communities in intertropical areas harbor greater pathogenic diversity than human populations living in subtemperate conditions (Fig. 33.8). In other words, the species richness of human pathogens follows the same pattern seen

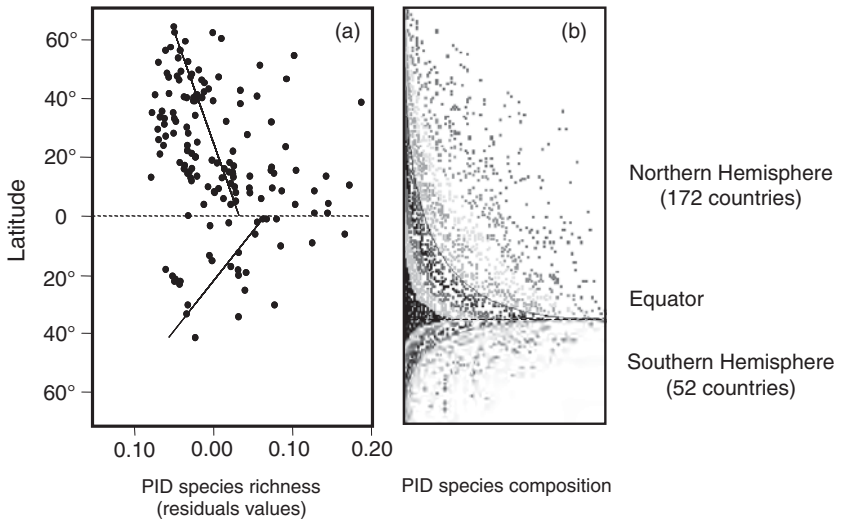

Fig. 33.8. (a) Evolution of parasitic and infectious disease (PID) species richness with latitude across the two northern and southern hemispheres. Dotted lines express the negative relationships between total species richness in human etiologic agents, after correcting for the effects of confounding factors on richness estimates, and the degree of latitude for the two hemispheres. (b) Presence/absence matrix for the 332 distinct PID species across the two hemispheres. The spatial distribution of PID species based on real data was run using a statistically rigorous method for model selection, that is, Monte-Carlo randomized permutations. The presence/absence matrix provides distributional information about which species occurs at each site (recorded by a point), and which does not. Figure (b) indicates that PID species diversity decreases as we move northward or southward from the equator, with pathogen species present in any human community living in a temperate area tending also to be present in richer intertropical human populations. Based on this test, the actual spatial distribution of human pathogens on Earth shows a clear nested pattern where some pathogens are restricted to the tropics, whereas others, more ubiquitous, are widely and regularly distributed throughout human populations. Such a distribution pattern provides information on the processes that are involved in generating the current geographical distribution and spatial range of human diseases. From Ref. [34]. See color plates.

in other free-living organism species [10,38,40]. But, to what extent, this geographical pattern might be the rule or the exception for such microbial human disease diversity? Many epidemiologists have treated this human pathogen diversity as a black box with no spatial structure, but it clearly appears that a large part of human microbes are not randomly distributed, notably those that are involved with multireservoir and zoonotic infectious diseases, and which strongly depend on the presence of their obligate hosts and/or reservoirs. One group of pathogens is obviously not conform to the general trend of spatial distribution observed, that is directly transmitted diseases specific to humans, which may quickly spread throughout the Earth with the potential to impact millions of people, and so which exhibits a high degree of homogenization. Many diseases like whooping cough and measles (see Chapter 12 in this volume) have made extraordinary evolutionary adjustments to coexist with humans over thousands and thousands of years, having originated in wildlife and then domesticated animals. Such diseases that have been around for a long time have extended progressively following humankind on its travels, migrations, and colonizations all around the world.

Clearly, many factors, often interacting with each other, can influence the actual spatial distribution and range of human pathogens. Thus, what is the relative importance of biological and socioeconomical factors? For human diseases, the causal geographical configuration seems obvious in that a large part of human pathogens originally comes from wildlife animals, a category that will continue to produce new infectious diseases particularly in the tropics where the biological diversity is the highest. The underlying initiating event of host switching from the "environment" as the source may then be amplified by some human social practices, human migration, continental and intercontinental exchanges that are circumstances that may facilitate the diffusion of microbes. However, it is difficult to attribute clear-cut impacts to one specific driver as recent emergence of new infectious diseases such as HIV, Lyme disease, and West Nile virus are due to human migration into new environments, specific cultural human behaviors or land modification as well [59]. Figure 33.8 gives an idea of the actual spatial distribution of human microbes in the world with most of species concentrated in the tropics thus in conformity with a common biogeographic rule [33], but the globalization of air travel and economic trades, global climate change, or land use and habitat modifications will accelerate the rate of human pathogens globally. The picture as illustrated in Figure 33.8 could be then progressively replaced by a new "global" picture where most of microbes are everywhere [59].

What the study by Guernier and collaborators [34] adds to our understanding of human disease biogeography is the knowledge that, quantitatively, their actual spatial distribution ranges, with the exception of the group of directly transmitted diseases specific to humans, strongly depends on climatic conditions. Again, this pattern is due to the strong associations that exist between indirectly transmitted pathogens such as dengue virus and the *Plasmodium* protozoan, or viruses causing viral hemorrhagic fevers, their vector or reservoir hosts, and habitat conditions. Undoubtedly, many human diseases are associated with environmental climatic conditions, and we might then ask how they will respond to the plausible range of global climate change over the coming five decades. This is exactly what the next section will deal with.

33.4.2 Enhanced Global Warming and the Spread of Infectious Diseases

The ecology of infectious diseases, and notably the manner in which hosts, vectors or reservoirs, and parasites interact with each other and their natural environment, represents a cornerstone in controlling disease, as global climate change could have far reaching effects on global patterns of disease distribution, with vectors, reservoirs and diseases once relegated to the tropics migrating to temperate zones. What Guernier et al.'s study tells us [34] that the influence of annual precipitation range and, to a lesser extent, of monthly temperature range is much more crucial than temperature and humidity *per se* in the occurrence and spatial range of numerous human parasitic and infectious disease agents. Examples of infectious diseases in plants and animals that have expanded their frequency and geographical ranges over recent years in response to partial global climate change have now been documented [37]. Indeed, one of the most convincing demonstrations of how recent climate change may intervene in health concerns lies in the incidence and frequency of cholera outbreaks in human communities in Bangladesh [76].

When analyzing historical data on cholera prevalence in Dhaka (Bangladesh), and *via* the use of sophisticated statistical procedures, Rodó and his colleagues [76] found a strong association between the *El Niño*/Southern Oscillation Index (SOI), a measure of ENSO, and the temporal dynamics of cholera. This signature was highly visible for the period between 1980 and 2001, corresponding to the well-documented Pacific basin shift of 1976. This signal was not visible or was too poorly pronounced to be detected for the period between 1893 and 1940 for which data were available. Figure 33.9 captures this tendency, during the more recent interval of time, of cholera population dynamics to oscillate with the SOI index, with strong correspondence of maxima of cholera to minima of SOI. From 1980 to 2001, a quasi-quadriennal cycle (a period of between 4 and 5 years) of more severe cholera outbreaks in Dhaka human communities was observed, and can be interpreted as being the result of a more prominent role of climate forcing by ENSO in cholera population dynamics during the last two to three decades. The consequences of climate change in terms of infectious diseases of both humans and animals (this is also true for plants!) are only now beginning to be evaluated and foreseen. The work by Rodó and his collaborators [76] is probably one of the first epidemiological contributions providing quantitative evidence of the impact of climatic change upon the interannual variability of an infectious disease (see also Ref.

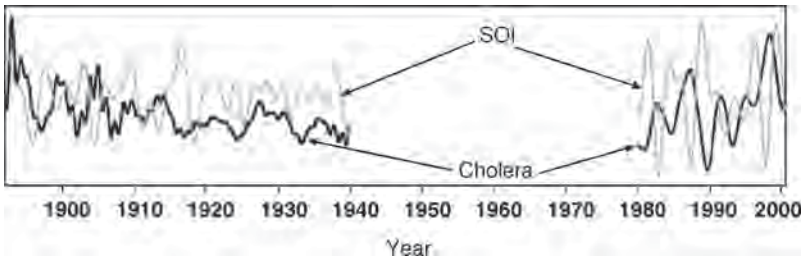

Fig. 33.9. Relationship between the Southern Oscillation Index (SOI) and cholera cases in Dhaka (Bangladesh). Data time series as illustrated in the figure are from the reconstruction of principal components based on original SOI and cholera cases in order to remove seasonal variation and isolate the dominant interannual variation in dynamics (see Ref. [76] for further details) [© *PNAS* (2002)].

[84]). The reader will find further details on statistical and mathematical techniques in Chapter 22 in this volume.

Interestingly, one study has gone a step beyond in the demonstration of an association between global climate change and disease. Viboud and colleagues found an association between the mortality and morbidity impact of influenza epidemics in France and ENSO oscillations for two independent influenza datasets over the period 1971–2002 [94]. The mortality impact of influenza in France was significantly higher during the 10 winter seasons with cold ENSO conditions than during the 16 winter periods with warmer conditions (Fig. 33.10). Another previous work also evidenced an important association between ENSO and hospitalization cases for influenza and viral pneumonia in Sacramento, California [18].

Although no biological mechanisms have yet been characterized to explain the influence of climate variability on both influenza dynamics and the amplitude of outbreaks in terms of number of cases affected by the disease, the authors argued that environmental conditions caused by global climate change might interfere with the emergence and spread of new epidemic flu virus variants, making them better adapted to surviving and propagating under prevailing conditions. Another important, nonmutually exclusive, group of arguments infer that local conditions associated with lower temperature and higher humidity rate, as observed during strong cold ENSO phases in Europe, might affect human body conditions, rendering them more prone to acquiring the disease, that is, *via* immunosuppression or individual behaviors, with more indoor crowding, thus facilitating flu transmission between individuals and across groups of people. There is at present no clear evidence for the existence of an evolutionary scenario of flu virus adaptation driven by environmental or climatic conditions. Some evidence suggests that rapid patterns of evolutionary change in viral antigen properties may be driven by intense selection from the host immune system itself, that is, antigenic drift [17], and the intervention of short-lived immunity that would act as a density-dependent constraint upon overall infection incidence [24], thus giving no or very little support today to the hypothesis of climate-driven selection in influenza virus diversity. However, a more complete explanation of differences between influenza virus variants in the future should also provide insight into whether climatic variability on a wide scale influences the survival of dominant flu virus variants.

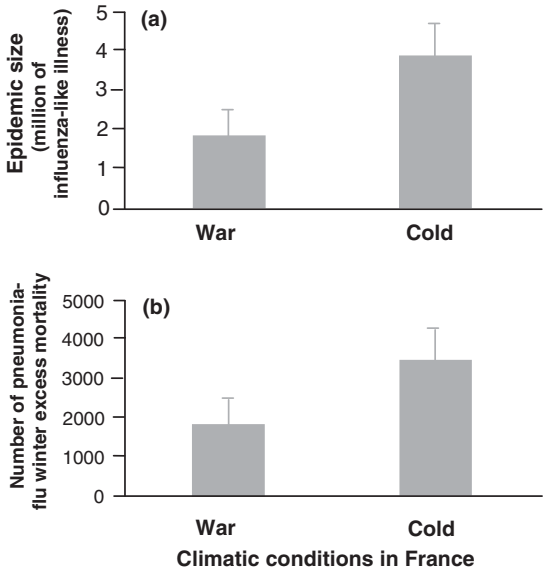

Fig. 33.10. Relationships between influenza-like syndrome morbidity (a) and mortality (b), and climatic conditions during cold and warm seasons in France. (a) Winter epidemic size average values and standard errors for 1984–2002. (b) Winter excess mortality due to pneumonia and influenza average values and standard errors for 1971–1997 (redrawn with permission from Viboud et al. [94]).

Man has long been aware of the fact that climatic conditions affect diseases, and there is much evidence for associations between climatic conditions and infectious diseases. As climate also has an effect upon host and parasite body physiology, host and microbe life cycles, their habitat, and numerous other environmental parameters, the unprecedented rise in temperature underscores the urgent need for developing appropriate research in order to understand adaptations occurring within the microbial world, and for predicting responses in the face of such anthropogenically induced changes. The reader will find further information in national and international reports (from Refs. [2,3,46], and the numerous references cited above), and a very recent book [63] on global environmental change and health/ecosystems issues.

33.4.3 Ecosystem Changes and Health

There exist many documented cases of infectious and parasitic diseases that illustrate the effect of ecosystem changes upon health. Relationships between ecosystem changes, including habitat modifications and host species imbalances, and human, animal, and plant health merit our close attention. Outbreaks of infectious diseases like Lyme disease, schistosomiasis, and hantavirus infections in Latin America are clear illustrations of how ecosystem modifications may strongly impact upon the emergence of new infectious diseases. Because much of this chapter is concerned with the links between biological diversity and disease risk, that is, the reduced dilution effect of biodiversity upon disease transmission (see concerned Section 33.3.2), we here illustrate this by two other examples of the influence of species composition within an ecosystem and habitat alteration, and the resulting shifts in host species.

Many infectious diseases have complex life cycles requiring a reservoir or vector host. One such infectious disease, schistosomiasis, constitutes one of the most debilitating tropical infectious diseases. These snail-transmitted trematodes are reemerging in different African and Southeast Asian countries despite the undisputable improvement in sanitary and socioeconomical conditions in these areas. Larval worms, called cercariae, leave snails and penetrate humans in contact with freshwater. Adult worms live in the circulatory system of humans, consuming blood. Their eggs lodge in various tissues of their final human hosts, leading to organ failure [13]. A key to this reemergence is the creation of habitats for the several species of snails that serve as initial intermediate host for human schistosomes. Snails proliferate in ricefield cultures, dams, and aquaculture extensions. For instance, large impoundments throughout Africa, notably construction of the Aswan Dam that created Lake Nasser, have substantially increased schistosome transmission, resulting in increased human morbidity and mortality [19].

Trophic cascades driven by species introduction may also favor snail population increases. One example is the recent reemergence of schistosomiasis in Lake Victoria in East Africa. An amazing species flock of endemic Cichlid fish had evolved in Lake Victoria, and a few highly specialized species, adapted to feeding on mollusks, along with the resulting low snail density, thus hampered transmission of schistosomes to humans. But, fishery biologists then introduced the Nile perch, *Lates niloticus*, in an attempt to stimulate the local economy. The Nile perch drastically reduced the abundance of native cichlids. The subsequent explosion of mollusk populations on the lakeshores and an increase in human settlements, which sought to benefit from the new fish economy in the area, created foci for schistosomiasis transmission [64]. Thus, introduction of the Nile perch resulted in both direct economic profits for local populations and the loss of biological diversity of endemic Cichlid fauna, thereby creating indirect human health problems over the long run.

Another good example of how hosts, vectors, and infectious disease agents interact with each other and with their ecosystem is that of malaria in the Amazonian rainforest. Deforestation due to intensified farming and agriculture and the trans-Amazonian highway construction provided the environment necessary for one specific mosquito, *Anopheles darlingi*, to flourish in disturbed habitats [14,89]. *A. darlingi* is known to occupy a particular ecological niche in the rainforest canopy, but habitat changes have offered new opportunities for this insect to rapidly adapt to lower habitat layers in these man-made open ecosystems. Human encroachments have thus moved *Plasmodium* protozoans into those areas in which mosquitoes were accidentally highly competent at malaria maintenance and transmission. Deforestation and its resulting ecological niche shift by free-pathogen insects, increased urbanization, and human migration are all implicated in the observed changes in malaria dynamics in the Amazon basin. Human disturbance of the rainforest due to intensified agriculture, proliferation of the highly important insect vector, and the introduction of the disease agent near human populations clearly show the intricacy of environmental and social factors contributing to amplification of disease spread. Changing environmental conditions like deforestation have also contributed to the emergence of hookworms, an important human pathogen in Haiti, for instance [51].

Over the past 50 years, industrial and agricultural changes, along with economic and social changes, rapid population growth and international travel, have inevitably contributed to changing the profile of infectious disease occurrence and distribution. At the same time, we have either forgotten or neglected the initial events underlying the emergence of infectious diseases. The complex dynamics of environmental and social factors should force us to take into account ecosystem approaches to human health programs in the very near future [74].

33.4.4 Land Use, Agricultural Development, Intensified Farming, and Health

In the recent history of our society, successive human settlements and encroachments, along with growing human populations at the planetary level, are requiring huge supplies of food for sustenance, along with land for providing essential ecosystem services to meet that need [4,70]. At present, these services are being disrupted by the "eruption" of a dominant species on the planet, that is, man, who needs more and more resources and facilities, often to the detriment of ecosystem stability and sustainability. The reader has free access to a series of seven very fascinating e-seminars on *Medical Ecology: Environmental Disturbance and Disease* by Dickson Despommier of Colombia University, USA at http://ci.columbia.edu/ci/eseminars/1111_detail.html.

What then are the ecological implications of agricultural land use and intensified farming and husbandry for human health? Are we in fact creating modern artificial ecosystems that may contribute to the spread of new invasive pathogens by breaking down natural barriers?

Patz and Confalonieri [70] listed the environmental factors associated with land use that may have an impact upon

emerging diseases. These include (i) agricultural development, (ii) urbanization, (iii) deforestation, (iv) population movements, (v) introduced species/pathogens, (vi) biodiversity loss, (vii) habitat fragmentation, (viii) water and air pollution, (ix) road building, (x) HIV/AIDS, (xi) climatic changes, and (xii) hydrological changes including dams [59]. In addition, land use for agricultural development, intensified farming and husbandry have strongly affected biodiversity and climate at rates that have no equivalent in the history of human societies.

Some examples of emerging infectious diseases due to land use changes and intensified practices in agronomy can be illustrated as follows.

The Japanese encephalitis virus has been a serious public health problem in many countries of Southeast Asia since the time it emerged in the early 1970s. The virus is a natural infection transmitted by mosquitoes of the *Culex vishnui* group as found in Indian ricefields, their most prolific breeding sites. Intensified rice paddies in the Tamil Nadu district, as in many other regions of developing countries, and the physical and chemical properties of ricefield waters, that may change in response to natural drivers or by anthropogenic actions, have strongly affected the abundance of culicines in ricefields [86]. The larval density of mosquitoes and their rate of development may depend on a series of biological factors, for example, dilution by rain, surface area size for cultivation practices, and agricultural operations, for example, use of fertilizer. With the development and extension of ricefield cultures, which represent the food staple for the many millions of people living in those areas, human-induced environmental changes have created foci for the development of vector and reservoir hosts. In particular, use of fertilizers with nitrate nitrogen has exerted a positive influence upon larval abundance of mosquitoes *via* a mediated-effect upon the multiplication of microorganisms in the ecosystem, which constitute the main diet of mosquito larvae. Similarly, in the same type of agroecosystem, but in northeastern Argentina, a strong correlation has been found between the abundance of *Biomphalaria* species, which are potential vectors for schistosomiasis transmission to humans, and, among other environmental parameters, nitrate and nitrite concentrations in ricefields due to fertilization [81]. Man-made creation of new habitat conditions in ricefields, through the addition of compounds such as calcium, the main component of the snail shell, has caused vector hosts to flourish, and humans usually contract the disease during the rice harvest *via* contact with infected snails. Similar scenarios have been observed for both Korean hemorrhagic fever, caused by a Hantaan virus, and Argentine hemorrhagic fever due to the Junin virus [61]. The virus responsible for Korean hemorrhagic fever is a source of natural infection in the field mouse, *Apodemus agrarius*, in many countries of Southeast Asia, particularly the People's Republic of China. Ricefield extension has created favorable conditions for the explosion of field mouse populations, thus increasing the risk of disease transmission to the population, and especially farmers. It is suspected that the conversion of grassland to maize cultivation, as seen in many districts of Argentina, has facilitated the proliferation of a rodent reservoir that is the natural host for this virus [61]. Additional examples exist of new areas subject to intensive cultivation, which witness the development of potential vector or reservoir host species [4,61]. Ecological changes due to agricultural development are among the most frequently identified factors in disease emergence, and it is important to consider that the need for more food supplies as human populations continue to grow will inevitably precipitate the emergence of new diseases, by placing more and more populations in close proximity to a natural reservoir or host.

Pandemic influenza is another illustration of how agriculture and intensified farming play an important role in disease outbreak. In general, communities are afflicted by annual or biennial epidemics of influenza caused by virus mutant strains highly selected to propagate in human hosts [17]. However, recent evidence shows that man, despite his relative resistance, may also be exposed to new influenza viruses from avian hosts [49,50], thus contradicting the more general belief that host switching is only occasional [62]. Waterfowl, such as ducks, constitute an important natural reservoir of influenza, but what is even more extraordinary is the high susceptibility of pigs to these avian influenza viruses. Recent findings [49,50] show that pigs might serve as "mixing vessels" [61] for the recombination of avian and human influenza viruses, thus generating novel influenza recombinants highly virulent for humans. It is now suspected that the ancient influenza pandemics might have resulted from the propagation of mammalian influenza recombinant strains [61]. But, what changes in human ecology facilitated the acquisition of avian influenza viruses by pigs, and the diffusion of new, highly virulent mixed influenza strains to humans? It now appears clear that influenza viruses benefit from a community of potential host species, rather than a single species, so as to evolve and select new forms better adapted to propagation. Intensified pig–duck farming, meant to provide food supplies to human populations, as is the case in Southeast Asia, has undoubtedly contributed to creating new "man-made ecosystems" [75] highly favorable to the diffusion and mixing of disease strains. As pointed out by Morse [61], these human influenza infections with complex zoonotic pathways have recently received much attention from public health authorities on intensified farming and in other settings, where different potential host species may be close together at high densities, as in live animal markets. Here we cite the recent outbreak of SARS in southern China, caused by a coronavirus from a small mammal,[1] which serves as a food source for the local population, as another illustration of the introduction of a new disease agent into human populations [31]. High-intensity production of food animals like cattle, along with rendering processes, have facilitated the transmission of the scrapie agent from sheep to cattle, causing bovine spongiform encephalopathy, later associated with a new variant of Creutzfeldt–Jakob disease in humans [26,93]. Intensified production of food and industrial

[1] A scientific discovery published after manuscript writing has shown that Chinese horseshoe hat was a reservoir for SARS disease-agent. See Li et al. (2005).

processes in our modern societies will clearly increase the incidence of accidental contamination and amplification, as observed for BSE, in which mammal by-products, mainly from sheep and cattle, were fed to cattle, thus forming a new "artificial food chain" and providing the opportunity for cannibalism to herbivorous cattle.

As human populations grow, agricultural development and intensified settings, as in poultry farms, will be important causes of both ecological disruption and interaction with the environment, thus providing suitable conditions for exposure to novel pathogens. There is now increasing concern about the many uncertainties surrounding the environmental origins of microbes; indeed science over the past decades has concentrated only on understanding the human side of this question. The flu epidemic in Southeast Asia is one recent illustration of the need to enhance our "window of knowledge" of the natural ecology of infectious diseases.

33.4.5 Human Population Growth and Behavioral Practices

Having discussed at length the imprint of humans on the Earth's ecosystems (see above), we will here concentrate on the potential effects exerted by increasing human population size and density on disease emergence and spread. In their respective conclusions, both McMichael [59] and Morse [61] agreed that current living conditions have created a situation in which the causes of disease emergence and spread are more prevalent than ever.

Indeed, the human population of the planet is now much greater than at the beginning of the twentieth century, creating a greater diversity of microbes existing in these increased populations [32]. Though we have little or no idea of how many pathogen species are hosted by human communities, it is now clear that larger populations may harbor a greater diversity of pathogens than smaller populations (Fig. 33.11).

Much remains to be understood on the mechanisms regulating the diversity of pathogens in human communities.

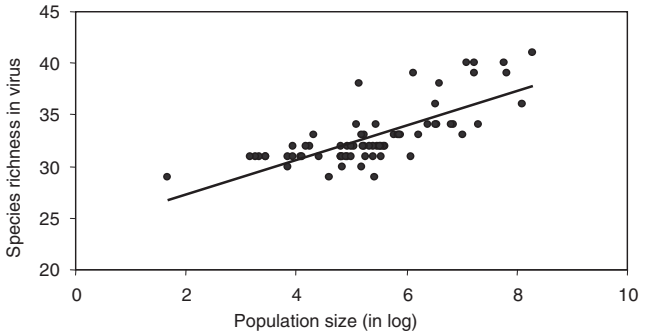

Fig. 33.11. Linear relationship between human population size and species diversity in viruses across 71 human communities living on different islands. Linear regression is $y = 1.67\ x + 23.97$, $r^2 = 0.551$, $p < 0.0001$. Population size variable is log-transformed. From Ref. [32].

The example above provides clear evidence of how the spatial scaling theory and community ecology rules might be best applied to pathogen microorganisms in humans. Many epidemiologists and public-health scientists have treated the "tiny world of human pathogens" as a "black-box" with no spatial structure or biogeography [25], but recent findings [25,34,83] illustrate that, like macroorganisms (see many references above) and other microorganisms, for example, microalgae, and fungi [27], human pathogens are not randomly distributed, but rather exhibit predictable spatial patterns. This offers exciting potential for a more synthesized view of human pathogen distribution and organization, and ultimately a new means of understanding and thus controlling infectious diseases.

Moreover, the effect of population size, that is, the number of susceptible persons within a community, on both disease persistence and spread is particularly important, and many recent studies have clearly shown its role in childhood diseases [5,28–30]. First, there exists a community size threshold, referred to as the critical community size (CCS), according to which disease probability extinction is high due to demographic and environmental heterogeneities. Thus, the disease can only persist with time in only human communities over a given population size, that is, around 250–300,000 inhabitants for both measles and whooping cough. Rohani and co-workers [79] showed that both measles and whooping cough (see also Chapter 12 in this volume) diffuse progressively from urban centers to the surrounding rural areas, with, for England and Wales, the three biggest cities, that is, London, Liverpool, and Birmingham, acting as sources of disease retransmission (Fig. 33.12).

Thus, many recent advances in the ecology of infectious diseases have led to the fascinating finding that numerous infectious diseases faced local fade-outs, but enjoyed regional persistence at the metapopulation level (see Chapter 12 for a definition), indicating that disease dynamics behavior in space and time cannot be understood without considering the importance of large spatial scales. Despite the public health importance of such findings, little is known about the regional influence of space in shaping disease incidence and prevalence within local communities. Major advances made during the recent decade in the metapopulation dynamics theory of infectious diseases clearly point to the fact that we need to adopt a macroscopic view of disease. It remains to be seen whether these ideas will be adopted in the very near future as our world progressively becomes a "small-village." We will deal with this in the final section.

33.4.6 International Travel and Trade

The historical processes that gave rise to the preponderant role of humankind on Earth are continuing. Humans, members of a common, ubiquitous species highly resilient to diverse habitat alterations, have exploded in size on the planet because modern living conditions have ensured their survival and reproduction. Modern technologies are a compound ingredient in the success of the "human saga," and have

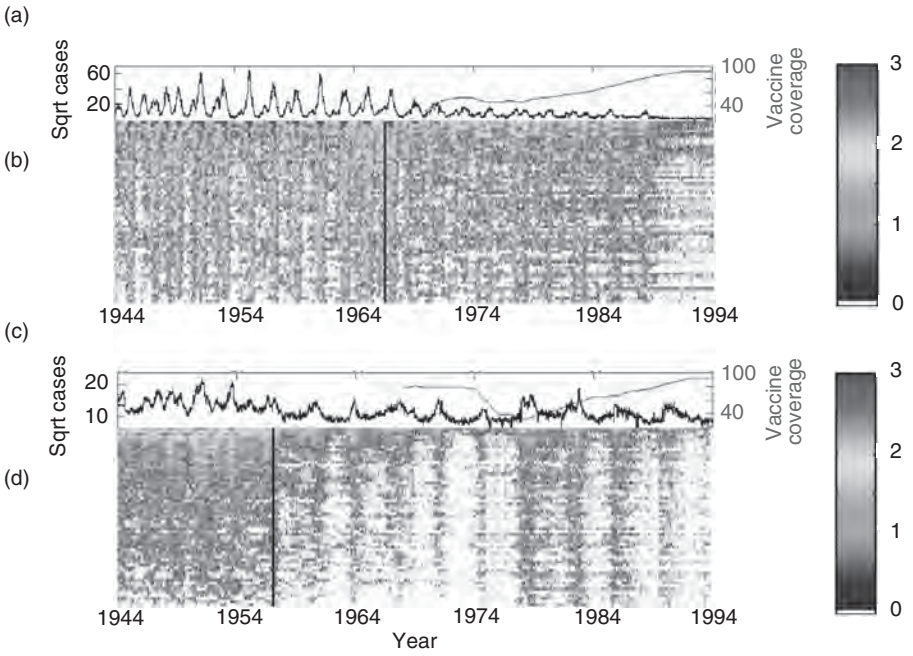

Fig. 33.12. a and c illustrate the population dynamics in number of infected cases for measles and whooping cough, respectively, for England and Wales. Gray curve shows the evolution of vaccine update for both diseases. (b) and (d) show the spatial patterning of number of disease cases ranked from the largest city, that is, London, at the top of subfigures to the smallest one, that is, Teignmouth, at the bottom of subfigures. Variability of gray (clear to dark) colors indicate the level of disease incidence. Spatial patterning on subfigures (b) and (c) indicates that both childhood diseases persist with time in the largest cities in England and Wales. This pattern is highly visible for measles on subfigure (b). Modified from Ref. [79] with permission from the senior author.

facilitated the transfer and encroachment of new populations into unoccupied areas of the world. Transcontinental air travel and maritime transport, along with global economic trade, have contributed to the success story, but evidence is now accumulating that supports the adverse effects of globalization upon the rapid spread of emerging infections and the diffusion of infectious diseases [85]. Today, speed of travel and global reach enable rapid access of disease to uninfected populations: Any population on Earth, with the possible exception of native tribes in the rainforest, is at risk of contracting any disease (Fig. 33.13). The recent avian flu epidemic in Southeast Asia and the world-wide panic it created is a crude example of the new artificial world we, as humans, have generated, thus facilitating the spread of infections [49]. Instead of choosing one or two specific diseases to illustrate this section, we present new findings here by Smith and coworkers, who have begun to quantify the degree of global homogenization of human diseases and have categorized the taxonomic groups of diseases at risk of contaminating human communities in the future [83].

Based on an extensive dataset of around 317 human infectious diseases affecting human populations on Earth, Smith and contributors [83] have categorized these different diseases according to the collection of reservoir hosts utilized: (i) infectious diseases specific to humans, (ii) infectious diseases that utilize human and nonhuman reservoir hosts (multireservoir), and (iii) zoonotic infectious diseases. Then, using a similarity index to measure the degree of homogenization across regions, the authors have revealed that infectious diseases specific to humans exhibit the greatest degree of similarity among nations and continents, followed by multireservoir infectious diseases, and finally zoonotic infectious diseases (Fig. 33.14).

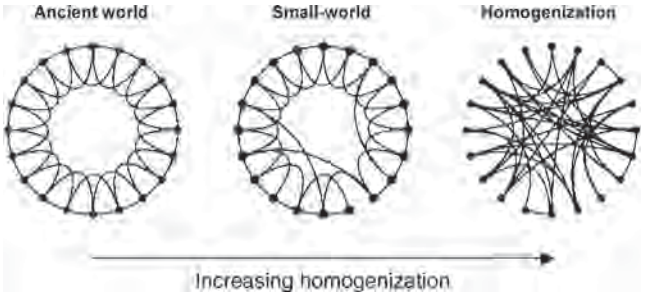

Fig. 33.13. Schematic illustration of (i) early conditions in which local communities were in contact with nearest neighbors only (on the left); (ii) small-world conditions (middle) where, in addition, a given local community was in occasional contact with another distant community, thus mimicking transcontinental exchanges; and (iii) global homogenization (on the right) where "any community is in contact with the others," illustrating a global world. Infectious diseases may benefit from this strongly interconnected world so as to proliferate and expand in host populations. Modified from Ref. [96].

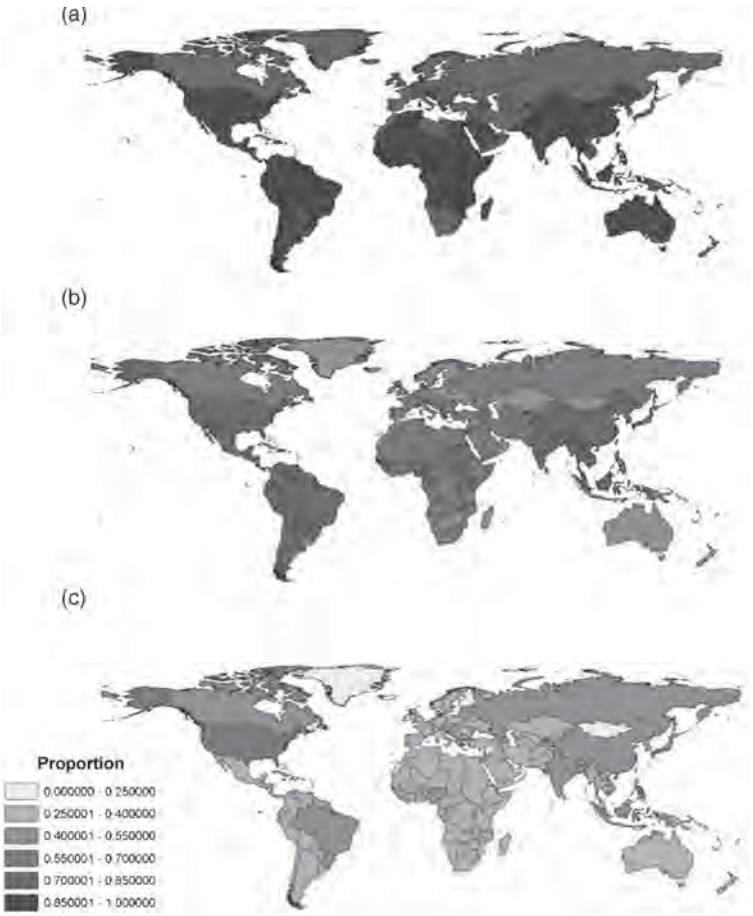

Fig. 33.14. Degree of homogenization at the national scale of human infectious diseases based on the range of hosts utilized: (a) infectious diseases specific to humans; (b) infectious diseases that utilize human and nonhuman reservoir hosts (multireservoir); and (c) zoonotic infectious diseases. The degree of homogenization ranking is based on Jaccard's similarity index from low (gray) to high (dark). With permission from Ref. [83].

At the national scale, the near global homogenization of infectious diseases specific to humans suggests that there is little scope for further expansion in their spatial distribution. However, although many multireservoir and zoonotic infectious diseases are also broadly distributed, far more remain localized to specific regions and localities of the world. As a consequence, infectious diseases with direct lifestyles represent the most likely candidates for spread in nations in which they are absent today. The spatial distribution of infectious diseases that utilize nonhuman hosts is likely due to the geographic distribution of their reservoir hosts. As in the case of the emergence of monkeypox disease in the United States, the increasing rate of introduction of exotic species will probably facilitate an increase in geographic scope of the infectious diseases they may host in the years to come. This raises a serious concern for public health, as pointed out by Smith and collaborators [83], how to target public health strategies, as human nonspecific infectious diseases might have the greatest potential for crossing frontiers and becoming established in new areas. Admittedly, this is not a new phenomenon. Humans have facilitated the spread of disease to novel and susceptible populations across the planet for hundreds, of thousands, of years [83]. The magnitude of these introductions has created a world in which many historically localized diseases are now broadly distributed and shared between regions. International travel and economic trade, particularly that of exotic pets (though it has since been demonstrated that the tire trade is also highly responsible for diffusion of disease vectors [75]), are particularly alarming, as they play a strong role in the diffusion of diseases in new regions, and as such, require immediate decisions when regulating trade and transport at the global level.

33.5 CONCLUSION AND SUGGESTED RESEARCH PERSPECTIVES

This chapter has explored and summarized topics on the links between global environmental changes and health concerns, and the problems and challenges posed by this new global context in terms of actual health issues. It is not, nor was it

intended to be, an exhaustive review of the numerous examples that now exist on the interrelationships between global changes and health. For instance, we deliberately decided not to include considerations on microbe adaptation and evolution, or the socioeconomic drivers of disease, as many chapters in this volume will deal with that matter. We also hope to have proposed an important list of references, which the reader can consult for further reading. Rather, we have selected topics that illustrate, for scientists, policy makers, and the public, the nature of scientific and health challenges posed by global change and implications from a global health perspective, giving "a broad picture" to show how health scientists, and national and international health authorities need to consider the complex nature of two-way interrelationships that truly exist between the environment and health.

Many academic epidemiologists believe that we have learned enough about infectious diseases, and this chapter tries to convince the more skeptical of them that we might take more important crucial steps in understanding disease by changing the way we work. The challenge for modern epidemiology is to open "the window," and to adopt a broader perspective on health. On page 166 of his book, Lawton has identified four areas in which he feels contemporary community ecology has got the balance wrong. These areas can be easily transposed to current epidemiology. They are (i) too great an emphasis on short-term, highly reductionist experiments or laboratory research; (ii) too great an emphasis on local, often tiny, processes at the expense of larger-scale regional ones; (iii) the absence of a connection between molecular ecology, population genetics, and population and community ecology of infectious diseases; and (iv) a nearly total failure in exploiting the power of model systems.

What then can we do to reduce uncertainty about the health responses to global environmental changes? Such an approach will require a shift in the way we operate as "epidemiologists," from field epidemiologists to computer-science epidemiologists. This volume may constitute a current demonstration that breaking with tradition in health can be a source of confusion, but it is also the means, and this was the intention of our scientific publisher, of bringing together different disciplines and subdisciplines for fruitful exchanges. What we need, then, are the following:

(i) *A plurality of approaches*: We need field observations, theory, experimental designs in the laboratory and in the field, hypothesis testing, molecular ecology, and global epidemiology.

(ii) *Fewer systems studied, but in greater depth*: We need to concentrate our research efforts on fewer places and biological systems in which to place our resources. As pointed out by Lawton, people hate this idea, but as two population and community ecologists, we can state here that health research will considerably benefit within the next years from this decision, and it is in the interest of epidemiology to adopt such an approach.

(iii) *Development of long-term epidemiological survey sites*: It is particularly true in tropical regions of the world where the impact of global environmental changes on health concerns will be the most substantial.

(iv) *Embedded into item (iii) is the need for standardization of protocols*: It is particularly true in different disciplines of epidemiology. Failure to standardize protocols over the past decades virtually destroyed any attempts at meta-analysis and comparative analysis in epidemiology. Curiously, the excuse of not being able to develop a comparative approach in epidemiology stems from the fact that no standardization of protocols has been decided.

(v) *Institutions and research*: There is a strong need on the part of national and international health institutions to promote more coordinated multidisciplinary research. The experience in the United States of a joint *National Science Foundation-National Institutes of Health* project represents an effort toward better collaboration between health scientists and those working on other fields of science.

(vi) *More nationally and internationally coordinated research programs*.

(vii) *Promoting community contributions instead of individual work*: There is a current tendency in epidemiology to consider molecular ecology as a "primer" in modern science, and to relegate other fields of research to second position. The same can be said for field epidemiology as related to theoretical epidemiology. We need to encourage a variety of approaches that will provide different points of view on disease.

Despite considerable research efforts made toward understanding patterns and processes that explain the occurrence, emergence, geographic spatial distribution, and extension of infectious diseases, epidemiologists are still not able to predict in detail how, where and when particular diseases will respond to environmental change. This represents a major research challenge for epidemiology in a changing world. We strongly feel that such a challenge can be met, widening our approaches and state of mind so as to join together in fighting disease.

33.6 SUMMARY

Recent studies of the impact of global environmental changes on the health of humans, and even wildlife, have provided several good examples of how large-scale investigations are of particular relevance to epidemiology. However, health study lacks both a broader "picture" and a comparative perspective, whereas major research developments have been made recently in other fields of life science, such as population dynamics,

community ecology, and macroecology, that have benefited from enlarging scales under scrutiny. In this chapter, we attempt an objective, though not exhaustive, analysis of global environmental changes and their impact upon infectious disease patterns. The analysis of large-scale global environmental hazards in epidemiology requires integrating knowledge of different disciplines, thus necessitating a holistic research approach. This chapter provides with numerous examples of how large-scale patterns may intervene with local-scale health concerns, and thus it tries to convince the more skeptical among public health-scientists and authorities that we might take more important crucial steps in understanding infectious disease by changing the way we work. Such a challenge can now be met, widening our approaches and state of mind so as to join together in fighting disease.

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CHAPTER 34

Contributions of Social Anthropology to Malaria Control

Jaffré Yannick

Ecole des Hautes Etudes en Sciences Sociales (SHADYC), Centre de la Vieille Charité, 13002 Marseille, France

34.1 INTRODUCTION

In order to triumph over malaria or at least reduce its morbid effects, malaria control strategies generally focus on three objectives involving broad fields of activity, each one unfortunately raising as many specific difficulties.

To begin with, a prompt and appropriate management of the disease strongly depends on its early diagnosis. Beyond the therapeutic benefits of such an approach, another key aspect of effective case management is the essential observance of the treatment. However, the development of drug-resistant malaria strains suggests improper or inappropriate use of treatments.

Next, it is also necessary to ensure the planning and implementation of selective and sustainable preventive measures, especially among highly vulnerable groups such as pregnant women. Insecticide-impregnated bed nets and curtains, in use since a few years, seem to be an effective means of preventing malaria. However, bed net usage is low in Africa.

Finally, implementation of national-specific programs should permit the reinforcement of local capacities in malaria control and research. Up to 80% of malaria endemic countries developed plans of action for malaria control but they are faced with the difficulties evoked above.

Thanks no doubt to health education campaigns and to an overall development of schooling, a real improvement of knowledge about the role of mosquitoes in malaria and the benefits of bed nets can be observed. However, these new understandings do not "automatically" result in new practices. Knowing is not doing . . . .

All the difficulties we have just evoked broadly explain the fact that even in countries where actions recommended by international organizations were applied, no real positive results can be observed either according to criteria of efficiency or measures of effectiveness. And, one must regret that "the burden of malaria has remained unchanged in malaria prone areas, particularly in Africa" [4].

34.1.1 A Poverty-Related Disease?

These gloomy facts cannot be considered in isolation and to understand this situation, it is first of all necessary to emphasize the importance of a global geographical, political, and economic environment.

Indeed, these hygienic situations are embedded in bigger social systems [56] and the use of a "distant gaze" reveals strong correlations between the characteristics of cultural and economic contexts and the prevalence of parasitic diseases.

It would not be amiss here to recall that malaria develops in contexts of great poverty. Thus, the African continent, particularly affected by this disease, counts 29 among the 35 countries considered to be of "low human development" with 22 being the last on the list [68].

Certainly, these figures cannot by themselves resume a human and cultural vitality that is itself difficult to quantify. In these countries, the harshness of living conditions demands an inventiveness that expresses itself particularly in the division of work and the management of informal activities.

But this continent, where the geographical context combines a natural environment that favors the development of numerous microorganisms with complex human data that are difficult to control—such as rapid urbanization, urban homes that are not adapted to the climate, family structures, complicated management of wastes [57]—adds to the difficulties (Fig. 34.1).

In sub-Saharan Africa—limiting ourselves to a few big indicators—the average rate of illiteracy varies between 20 and 50% and the flat rate of school going girls is the lowest in the world [44]. Yet, several studies emphasize that the level of women's education brings about a fall of prenatal and infantile mortality [13,22,46] and a better understanding of health proposals [7].

Besides, drainage structures and availability of drinking water are widely insufficient and access to health services remains uncertain for the large number. This set of political, economic, social, and cultural relations—that some group under the term "political ecology of disease"—constitutes a complex whole

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

Fig. 34.1. A house in the town of Niamey (Niger) (IRD/ Indigo/Photo Sabriè Marie-Lise). Health program must not be a dream or a sheet of paper but must be linked with real society.

that forms the ground on which malaria develops. Simply speaking, to be a medically defined disease, malaria, like many of the parasitic and infectious pathologies (trachoma, tuberculosis, diarrhea, dermatomes, or acute respiratory infections) is a disease related to poverty and revelatory of inequalities [1].

These pathologies result largely from human forms of coexistence and the dialogue they maintain with an environment that they progressively transform. The change of ecosystems, the modifications of social relations, and the state crises construct the variable contexts for the emergence and diffusion of these diseases.

For example, the transmission of schistosomiases—and also of malaria—is partly linked to the realization of hydraulic works [28,62]. Migration toward urban areas—by imposing periods of celibacy and reducing social control—favor the growth of MST and AIDS [45,64], and these shifts can negatively influence the rate of immunization [42]. In extreme situations, there have been 27,000 victims of exanthemata typhoid in refugee camps in Burundi between October 1996 and May 1997, and "the revival of African human trypanosomiasis is an indicator of chronic political crises that have had a destabilizing effect in all fields. There can no public health without peace" [1, p. 33].

Such commentaries—as well as researches of historical epidemiology showing that the important fall of mortality registered since two centuries thanks to the retreat of major infectious diseases, particularly tuberculosis, took place before the perfection of effective therapies [6,51]—invite attention to the importance of global responses in terms of development aid.

Health promotion thus reduces the risk by improving a coherent delivery of "public goods" (agriculture, housing, education, administration), which are its principal determinants.

Similarly, it is important to fight against the increasing signs of inequality, especially in the access to treatments [15,18]. It will suffice here to evoke the importance of relaxing the patent rules on medicines.

These questions, though "vast" are no less crucial because this global economy affects the most intimate levels of life and the daily management of health. Indeed, in worlds where the state—or a collectivity built and directed by a stable set of rules is applicable to all—cannot ensure a minimum of social security, the individual is helped only in proportion to his direct participation in "natural" communities—family, neighborhood, colleagues—exercising a function of "close security" [37].

The practical consequences of this situation are important: The possibilities of treatment depend most often, on interfamily help that must be solicited for every pathological episode. This dramatically haphazard aspect of health care and the observance of treatment are responsible for the late arrivals in health services, therapeutic failures, and resistance to taking medicine [19,32].

Otherwise and broadly speaking, in many a case, parasitic diseases reveal a crisis of under development and at the very core of suffering, this confrontation of technical possibilities offered by industrial civilizations with those of the so-called "South" are an unbearable misery.

> I am always affected. The problem is the care. The treatment begins and there is no regularity, it hurts. One can't tell the patient he will be cured. On can do only what is possible. All through the day, diagnoses are made but the prescriptions can't be bought. I feel heipless; one does the necessary, but not the useful. (Malian nurse and intern, in Ref. [33])

Our world adjoins and now includes these practices. In addition, the persistence of unequal economic exchanges and demographic tendencies underlines how "futile it is to guard the illusion that the wall of prosperity and technical strength will protect the deprived people, that revolts of misery will be kept at a distance" [5].

The conclusion is obvious: The main response to this medical problem is social. In particular, it turns around the State's existence and efficiency and the persistence of nonadministered geographical zones [8].

Therefore, a minimum of aid to developing countries can be considered as a kind of necessary "insurance contract," ideally as an application of a "politics of hope" that does not characterize continents only by a lack of capital, equipment, personnel, jobs, initiative, and so on, but underlines the fact that for the Third World countries "the necessity of not being a poor imitation of currently equipped societies is as urgent as the necessity of being better" [5, p. 136]. Concretely, it is more important to codefine and coproduce health actions than to initiate them—even impose them—from "outside," according to abstract models.

Considering "concrete realities" of the field and elaborating projects between real partners are two indispensable criteria for the application of research programs and for an adapted and durable health development.

These observations emphasize that the "constituent" causes of the disease are not limited to an exclusively medical definition. But, what are the operational consequences that can be drawn from this vast anthropological perspective?

34.2 SIX PROPOSALS OF RESEARCH AND CONTROL

Faced with these difficulties, anthropology can give no lessons and still less "denounce" the courageous work often undertaken by medical teams. On the contrary, this discipline can help to resolve the difficulties encountered by the programs, by a different analysis.

First, the use of qualitative tools should permit us to see how laypeople perceive and classify illnesses in their own languages and belief systems. Next, this could help analyze the reasons behind the conduct of social actors: If they have no medical reason for acting as they do, they nonetheless do not act without "reasons." Finally, the social sciences are committed to understanding the behavioral logics of populations, which can differ from the medical perspective, by looking at health-related behavior in relation to larger social and political constraints.

If we agree upon these broad hypotheses and anthropology's special position of thinking about questions from the others' view point, reasons, and constraints, it seems possible to distinguish six kinds of goals corresponding to the questions as much as to the larger proposals of actions.

First proposal: Knowledge of population groups is essential for health care. Consequently, in order to improve access to care and observance of treatment, it is important to understand and analyze popular systems of interpreting illness.

A large number of researches in social anthropology have attempted to account for laypeople's beliefs regarding malaria-like symptoms and understand how language and the underlying cultural context of this disease reflects and spills over into this pathological field according to specific characteristics. True, these works only refer to ethno linguistics. However, this approach is useful for public health practitioners because it permits the constitution of glossaries of perceived pathologies linked to vernacular terms with symptoms that can evoke malaria from a medical point of view. How can a dialogue or clinical encounter be conducted without any understanding between the patient and the health professional?

This work of translation is crucial even for native health care professionals, given the important and inevitable gaps between popular and scientific systems of interpretation of the disease [2]. Indeed, medical nosology is largely etiology-based; therefore, some syndromes can group apparently different symptoms. On the contrary, "popular knowledge" applies itself preferably to what is visible and perceived. Identical disorders are designed generally by a similar illness term. Classification is then principally of a semiological kind and often misled by the complexity of the pathological processes.[1]

> Malaria is a fever and also an illness. I can say that it first starts as a fever and then becomes an illness. At first, one has a headache and the body becomes hot. But if you don't treat it at that point, it will become an illness (. . .). There are three types of malaria. With the first type, the eyes become yellow. In the second type, the eyes turn white and in the third, they turn red (. . .). If you catch the white type, your ribs and back become stiff. You also have headaches, over-heated feet soles and constipation. . . .

A large number of works on West Africa illustrated the gaps between semantic fields of scientific discourse articulated in French or in English and lay concepts expressed largely in African languages, which, not compelled to name modernity, have not developed a scientific and technical vocabulary for it.

For example, in Burkina Faso, Bonnet underlines that Mooré nosology classifies disorders that would be indicative of malaria symptoms from a medical point of view under several pathological entities (hypothermia and headaches, hepatic problems, and nausea) [10].

Beyond this understandable focus on the expressions of the disease, popular empiricism also leads to the classification of complaints by their frequency in time and season. In Niger, the local term for malaria would be *heemar ize*, literally signifying "the son of harvest time" in the Zarma language. *Sibidu* is the Wolof term in Senegal and conveys the idea of "a return of the illness." Among the Bambara in Mali, some fevers are thus named "sumaya," literally "coolness" [30,60].

Faced with these symptoms, there are various popular etiologies that offer causal explanations. However, these beliefs are mostly "prosaic" and empirical, based on what is visible or felt. For instance, when the main "reasons" of the illness are explained as being the result of excessive consumption of greasy, oily, heavy, or sweet foods like mangoes, they are preferably derived from the physiological dimension. The sensations of nausea related to the life cycle of the parasite are not considered here as the effect but as the cause. More marginally, ecological changes linked to the rainy season (smell of young millet) are also put forward and sometimes by "opening onto" socioreligious questions, so is the child's frail body and "soul" or "double."

> When a mosquito bites a sick person and then bites you later, it transmits the disease to you. Malaria can also be caused by the diet. Some foods are responsible for provoking malaria in persons without them being bitten by mosquitoes. These foods include many fruits like the shea fruit, banana and many others. If you eat a lot of eggs, you can get malaria very easily. (. . .) It is the battle these fruits lead against your body temperature that you won't be able to tolerate and that's what will cause malaria.

Finally, the process of aggravation of these pathologies is considered on the lines of a "hardening" of the initial disease. As the disease progresses and changes, there can be a shift in its terminology: for example, the local illness term *sumaya* can become *sumaya kogolen* (hard *sumaya*) or even evolve toward *sayi* (icterus).[2]

[1] For example, how can different symptoms of syphilis refer to only one term or how can one not confuse some joint pains with those caused by drepanocytosis.

[2] See Jaffré and Olivier de Sardan [36] for issues concerning laypeople's conceptions on illness nosology, physiology, and classification in West Africa.

> Fevers in a way, because they can cause diseases. When they "settle down in you" (*k'u basigi*), that's when they lead to other ailments. The one which moves in the body is by far the worst for it brings with it sickness everywhere it goes. Sometimes, it can even reach the veins of the heart (*sonjuru*), causing death by stopping the heart beat.

In brief, because malaria does not have a distinctive symptomatology, the differences between local disease labels and causal explanations given by scientific knowledge are particularly marked.

These illustrations are not wholly exotic or scientific. In concrete terms, these sociolinguistic differences have significant health consequences.

First of all, as malaria symptoms like fever, chills, headaches, and joint pains are common to other harmless childhood disorders, they are labeled in the same category and thus perceived similarly. To put it simply, these frequent childhood illnesses as well as those referring to "the hot body" usually end well [23]. Globally, only 1% of malaria-caused fevers progress to acute malaria illness [11]. Thus, it makes these infections seem socially quite commonplace.

> One can catch a fever by tiredness. After a hard and tiring work, one can, because of exhaustion, catch these fevers. Strenuous physical exercises can produce fevers. If you run or walk too much, fevers can occur. (. . .) That's what we call general tiredness. It's a sickness that doesn't kill but makes you exhausted. The sick child or person is bedridden and can't do anything.

Secondly, the distinct symptomatology between febrile illnesses and acute malaria-associated convulsions explains why people do not perceive these symptoms as the expression of a same biomedically defined disease ranging from mild to severe malaria. In African languages, this separation is confirmed by the existence of different terms to qualify an "ordinary" fever and convulsions; for example, in Bambara, *kònò, which* relates to convulsions, is distinct from *sumaya*. Besides, because of their phenomenology and the importance of interpretive systems using analogical reasoning, the latter are often attributed to natural entities (such as birds because of the flapping of wings akin to trembling) or to supernatural forces (spirits or devils, the capture of the double), because possession is often accompanied by visible paroxysms.

These different linguistic illness labels define health-seeking behaviors and explain why people do not worry at the outset of fever. The population's access to biomedical health facilities depends on these representations of the disease and the popular physiological conceptions that make up notions of risk, severity, and illness progression.

Because of an absence of scientific analysis of these lay interpretations of the disease and treatments, confusions are more the rule than the exception among health teams and populations. In the case of malaria, the polysemous aspect of the fever widens the interpretation gap between popular and medical conceptions. But one could also evoke trachoma, only perceived in the final phase of trichiasis, AIDS, the "new disease" expressing itself in the form of other diseases, or tuberculosis, often confused in its beginnings with a simple cough. Or, the people can only be concerned about preventing that which they name and understand.

Hence, a prerequisite of health education is to make medically identified diseases exist socially, before attempting to associate them with a preventive behavior or with the offer of treatment.

Indeed, if one can "undergo" or benefit quite mechanically from a prevention campaign, adhering to a preventive act—making the effort of pursuing it over time—implies knowing which pathology is "targeted," believing in one's own vulnerability, understanding the seriousness of the disease, and being persuaded of the effectiveness of this preventive act [27].

Initiating this educational dialogue is a difficult task for a country like Africa, which for example, counts 800 languages. But, "there is no chance of dialogue without understanding," and a health team cannot hope to open a real exchange with populations without including this work of "applied ethno linguistics" within its activities [36,49,66].

Second proposal: Health-seeking behaviors correspond to choices among "available health care options." It is therefore necessary to know them in order to improve patient's access to health services.

Health care behaviors are therefore largely governed by popular systems of interpretation of local illnesses. But, this initial set of characteristics combined with others, principally the multiplicity of health care options, explains the specific conduct of populations.

It is commonly observed everywhere that mothers not only give health care to children in terms of hygiene and nutrition but also often administer home-based remedies. Therapeutic products being sold over-the-counter, families use available medications such as "herbs" or leftovers from previous illness episodes to minimize care expenses as much as possible. Home-based treatments are all the more common for being an affective dimension of financial trade-offs and guide family economies. For example, the social meaning of a request for money can largely outweigh its simple exchange value and express rivalries between cowives trying to bring up and look after their children without "bothering their husband" with constant health care needs. For these reasons and others from the same social sphere, one has therefore to "manage with the least expenditure". . . .

Therefore, open-air pharmacies are largely used. For the population, everything argues in their favor. In fact, people favor this informal sale of medicines because of the mutual understanding between sellers and buyers with regard to illness labeling, easy access, immediate delivery at apparently lower costs, and greater autonomy in the patients' conducts [31].

> My last malaria episode was of the yellowish type. I said nothing to anybody. I went to look for roots of *nkankoro* [*Strychnos spinosa*] that I boiled for a long moment; then I drank the root decoction several times for two days. The third day, I started feeling better and that's how I could cure my malaria. I didn't say anything to anybody. I'm careful and I do both injections and drink decoctions . . . .

These practices appear harmless because they occur during the course of daily and ordinary affairs. However, in 1999, three markets in Bamako, on their own grouped 197 salesmen, proposing on an average 50 kinds of "pharmaceutical" products, whose prices ranged from 50 to 200 Fcfa. The methodical observation of the sale realized by these "informal chemists" allowed us to make an average estimate of the business turnover at 3500 Fcfa per hour and per salesman. If these estimates are agreed upon, these sums represent a daily exchange of around 10 million Fcfa and therefore that means an annual monetary flow of more than 2 billion Fcfa [31].

Besides, the informal health sector includes more than traditional healers. It also has a substantial "modern sector" composed of medical students, unemployed doctors, nurses, or even any person having had a contact with the health sector and knowing how to give an injection. These well-known and accepted illegal health actors make frequent use of injections and perfusions without any previous diagnosis. They take financial advantage of an important social demand, which rates the "direct treatment inside the body" highly.

These popular conceptions of the body and the effectiveness of treatments generate a strong social demand that finds an answer in a "neo-traditional" offer of health care. For example, at Lomé, Togo's capital, out of 1044 "health" centers only 11% corresponded to the "modern" medical sector. The other places of health care consisted of an informal medical sector (42%), "herbal therapy" (15%), divine healing (16%), protestant pastors of various denominations, various kinds of healers (9%) . . . [61] (Fig. 34.2).

This uncontrolled therapeutic pluralism is constant in all developing countries and it is important to emphasize how much these illegal practices have significant health consequences and how this "popular way of pulling through" enhances problems of chemoresistance.

Fig. 34.2. An example of informal medical sector: herbal therapy for malaria (IRD/Indigo/photo Bourdy Geneviève).

The patients' health itineraries combine all these constraints with coherence and pragmatism. These behavioral logics are simply the result of negotiations between illness causation beliefs, economic and affective constraints, uncertainties of health care options, and mobilization of cognitive categories that acknowledge the illness and its progress. In terms of public health, such observations indicate that these everyday behaviors will not be modified only by loquacious "sensitizing," but because other more economic and socially adapted solutions are proposed.

In the countries of the North, the state ensures an insurance structure principally in the form of a public service providing a majority with essential goods that cannot be the responsibility of private interests [17].

To ensure equal health care, the question arises of knowing which form of social security can be applied in developing countries and many experiences—from associations to systems of community health—are experimented, often at a local scale [12,16].

This economic dimension is fundamental because it can bring some permanence in health and preventive activities. But also if "the individual is to really make projects, establish reliable contracts, he must be able to count upon a foundation of objective resources. In order to plan in the future, a minimum of security is essential in the present" [14, p. 76].

Even in situations of poverty, the improvement of the offer of health could incite populations to transfer resources often used in social ceremonies (funerals, baptisms, dowry) to health. Therefore, the question is as much of pecuniary and material help as of perceived quality of health care and therefore of restoring recognition and dignity in health care centers. Foreseeing health risk is also a question of moral economy [41,63].

Third proposal: Health-related conducts are not from these actors' viewpoint, conducts that are health promoting. Therefore, the adoption of preventive measures depends upon a set of factors that are not only medical but also social.

Many popular practices exist to avoid the nuisance of mosquito bites (and obviously not only the *Anopheles* mosquitoes) such as fumigation, burning green leaves on the hut's threshold, mosquito coils, insecticide sprays, and repellents. These methods aim at the visible and the perceived nuisance.

From a medical standpoint, the main protective measure proposed is bed nets, possibly impregnated with insecticide. However, in order to understand its usage, it is necessary to describe how this technical innovation is embedded in bigger affective and behavioral wholes like a kind of transplant that "takes" or is rejected, most often by transforming its original structure. People reorient popularized recommendations, mainly because health-related conducts having an impact on health are not, from their perspective, health-promoting behaviors. Medical advice on prevention therefore comes to be integrated in a set of behaviors guided by other types of rationality.

> At home, children must sleep with old people like grandmothers and grandfathers. Those are the ones who wish to have a child beside them. Therefore, let the children sleep next to them. (. . .) If you have the money, then you can buy a large-size bed for three persons where they can sleep even if there are four or five children. If you don't have the means, then buy mats.

Thus, and rightly so from the health point of view, malaria control programs recommend bed nets. However, from a social point of view, this is a matter of "bed manners": schema of incorporated action, cultural norms that rule sleeping arrangements and justify the way of sleeping or sharing one's bed in a certain way. For example, in sub-Saharan Africa, sleeping, language, and kitchen manners are linked [58]; and among polygamous couples, the woman who cooks will also be "of bed." Likewise, a child who is sleeping is watched over and protected. The child often dozes on a mat outdoors next to his parents during the evening and then sleeps with his mother when night has fallen.

> Children who do not yet distinguish between the mother and the father can sleep with their parents. But as soon as the child is four or five years old, he should no longer sleep with his parents. If you insist on sleeping with a child of that age, it can happen that if he wakes up to have a wee, at that very moment you could find yourself engaged in sexual intercourse with your wife . . . . That is why when the child reaches a certain age, you must make sure that he sleeps elsewhere, either with his grandmother or with his older brothers and sisters under the veranda or in another room.

It is also a question of ways of using space. In rural Africa, the room and the bed are not always "autonomised" spaces corresponding to a specific activity. And, if in Europe, every activity has a corresponding space and in Africa (in the rural milieu) the same space can often fulfill overlapping functions. Thanks to regular sweeping, one eats, cooks, and one can sleep in the same place.

And more simply, the family size, the number of persons per room—(sometimes families of more than 50 persons live in a single housing unit with more than 10 persons per room) and the recent use of sheet metal roofs ensure that one cannot sleep inside and especially not under a bed net.

> Usually, a bed is made for two or three persons, but if the house is too small and the family too large, one cannot fix a precise place for everyone to sleep (. . .) I do not use bed nets because that would be too expensive for me. And then, I would be obliged to have bed nets for all the children although the rooms are narrow. To avoid that, I use mosquito coils. (. . .) And then apart from the expenses, if we all had to use bed nets, it would fill the entire room and there would no longer be any space left to cross it. It bothers you especially when you want to go to the bathroom.

In short, progressively, affective reasons related to kinship, to the child's status or to local architectures deconstruct and wear out the theoretical coherence of health-related messages. Proposals of prevention are reshaped by everyday life

Fig. 34.3. Bed manners in Sénégal (IRD/indigo/photo Paris Yves).

experiences: Children tearing up nets while playing, hot weather that makes it uncomfortable to sleep inside, sexual privacy which requires children to be kept away, use of slat beds through which mosquitoes sneak in, birthright that grants bed nets to elders . . . (Fig. 34.3).

For a better insight into these questions, it is necessary to study these microarrangements that for instance link economic wealth, the power to act and schemas of action in their contexts. Behaviors are more the result of juxtapositions of these contradictory constraints and diverse "collusions" between norms of behavior belonging to different social fields than an innocent consecution of acts through their representations.[3]

These everyday norms and gestures identified as "habitus" create a way of life. And that is why insecticide-treated bed nets are used in the framework of limited programs—when the project plays the role of a reminder of the new norms proposed—but their effectiveness diminishes when the new gestures that this innovation imposes are eroded, swallowed up by the automatisms of daily behavior.

> Today, you can get transparent bed nets which allow people to see through. I prefer those made of opaque fabrics, which are a bit dark inside. That is the most protective. No one can see what you're doing inside and they can also protect you from mosquitoes.

More generally, it is therefore not only a question of information, understanding and individual will. Knowing the preventive measures does not automatically mean accepting these or being able to implement them. The adoption of new behaviors always implies an invisible negotiation between various constraints (economic, cultural, familial, etc.) and representations of disease (Fig. 34.4).

[3] In the European sphere historians like Vigarello [65] emphasize that "the hierarchy of categories of reference must be overturned: it is not the hygienists for example who lay down the criteria of cleanliness in the seventeenth century but the authors of books concerning rules of propriety, practitioners of good manners and not scholars."

Fig. 34.4. Shrimp fishing with bed net (IRD/Indigo/photo Moizo Bernard).

These questions are at the heart of preventive practices. For example, in another context of dermatology and especially scabies, dialoguing with populations implies articulating a normative conception of hygiene (promoting hygiene, battling against promiscuity ...) with a comprehensive attitude toward behaviors and local life styles: body anthropology and "bed manners," cultural modalities of shame, modesty, and so on [25]. Further, many interruptions of treatment (TBC, HIV) are understandable because of social stigmatization that leads to hiding one's state and therefore distancing oneself from health services when pain does not prevent community living [24]. Finally, more practically, the necessity of cultivating rice overrides that of preventing bilharziosis (Fig. 34.5).

In most cases, risk therefore corresponds to an attempt at reconciling contradictory orders—hygienic, economic, affective, and others. Hence, rather than defining "vulnerable populations," it is important to understand which agencies can construct "vulnerable contexts" and lead—even force some populations to become "vulnerable." The danger does not correspond to a wish, or a false conception, which it would suffice to prove wrong, but results from complex arrangements and efforts to resolve contradictory orders.

Fig. 34.5. A new place for ænophilus in rural place in West Africa (IRD/Indogo/Photo Gazin Pierre).

That is why medical action must include a descriptive work of contexts of intervention, combining studies that allow a quantification of behaviors with others that deal with meanings given to them by their authors. These anthropological studies describing both the "objective" risks and their social interpretations should allow us to propose behavioral changes which have not only an epidemiological effect but also meaning for populations.

Fourth proposal: Treatment observance being related to the quality of the relationship between caregivers and patients, it is essential to analyze not only the objective but also perceived quality of health services.

Since some years, anthropological studies on the basis of precise descriptions have highlighted that various violent practices deteriorate the quality of the relationship between the population and the health care personnel [38,40]. Lengthy and useless waiting, carelessness, hasty consultations, and regular corrupt practices are unfortunately common practices in health services, which thereby appear to patients as "inhospitable" places [37,40]. "The patient enters the consultation room and is asked the reason for his visit; he reports a headache and aspirin pills are given without the least medical anamnesis or physical examination; besides, measurements of consultation time frequently show that cases are often seen in less than a minute" [20]. In this dialogue of the deaf and blind treatment, how can it be imagined that the prescription is relevant and scrupulously followed?

Far from being marginal, this question is at the heart of the therapeutic action. Thus, a survey carried out in 1994 in 40 health centers of Ghana, concerning 3950 patients, underlined that for 70% of the consultants treated for fever, the temperature had not been taken [52].

But, if the diagnostic approach is thus carried out "approximately" and by clinical error, the therapeutic response is on the contrary carried out "zealously." Indeed, all the patients were treated with chloroquine, the number of medicines prescribed varying from 1 to 12 for each of them. Eighty-five percent of all the patients, principally children, had received an injection. In short, in the majority of cases, treatments had been prescribed blindly, without preliminary diagnosis and with important iatrogenic risks linked to the injections [21].

Another study realized in the years 1991–1992 in Senegal, Dakar, underlined that of the 218 diagnoses of children evoking malaria, 70% were mistaken [21].

In a different context of Malawi, only half the children and adults appearing in health structures with symptoms of fever had received an antimalarial treatment, without there being any clinical justification for it [48].

Far from being exceptional, this lack of precision in diagnosis is therefore frequent and besides the questions of training and professional ethics is largely explained by the polysemous aspect of the fever. However, if in tropical zones, all

fever can evoke malaria, this cannot mean that every fever is malaria, or that every sensation of a "hot body" be interpreted as being a fever.

Other than these obvious therapeutic aspects, these numerous inaccuracies can in certain cases heighten the cost of care of the current pathological episode. They can also, by observation of the repetition of the same treatments and sometimes by the affirmation of their ineffectiveness, result in a pejorative opinion of the health services.

Confronted with these questions, it would not help to "address complaints" against health personnel. On the contrary, it is important to understand the reasons for these behaviors in order to improve them—low salaries, lack of equipment, impossibility of being responsible for "all the world's misery" [35], and others . . . . But in terms of public health, the extent to which these practices are harmful to patients and alienate them from health services, must be emphasized. Equally, how, besides sensitizing populations, it is crucial to help in the construction of a deontological guideline. Improving the care of malaria patients implies a global improvement of the quality of the health system.

Fifth proposal: Every health project addresses specific aims. However, given that countries or community groups benefit from several malaria control programs, one cannot understand these actors' "*responses*" *to health proposals without analyzing their synthesis of the numerous goals whose* "*target*" *they are.*

Let us be realistic and underline first of all that just like in those parlor games where the message is transformed and slowly loses its meaning during its transmission from one person to another, so are broad objectives defined by international organizations or by national programs translated by a set of simple and regular actions on a national scale: education of trainers in control strategies, elaboration of studies for national policy design, organization of seminars, training of agents in techniques of impregnation of bed nets, popularization of bed nets and curtains . . . . These activities often become "routine" and thus lose both their power of conviction and the sharpness of their initial objectives.

As everyone tries to survive, these activities are included in individual and economic strategies aimed at maximizing the resources of the NGO personnel or the administration. These phenomena of search for gains that projects procure sometimes disrupt health services by inciting health personnel to propose their skills to different "counters." Development policies can lead to pernicious effects: projects often "verticalize" action at the cost of a more global perspective.

Furthermore, every program seeks to achieve objectives that are often defined, and rightly so, from a medical and epidemiological standpoint: the vulnerability to certain aspects of the illness and implementation of preventive measures . . . . This perspective is legitimate and essential. However, if programs do suffer from amnesia and are designed on their own, populations do not forget earlier projects or the attitudes of their personnel, promises made and sometimes not kept. A great many "responses" of communities can be explained more by recent experiences than by the so-called "cultural constraints."

Similarly, a community often constitutes an arena for several projects going on at the same time (AIDS, diarrhea, nutrition, immunization malaria, etc.) without there being any authority to assemble the mosaic of their various actions and health proposals in a coherent manner. Populations therefore have to put everything together by themselves. They often do it in the form of syllogisms, for example, when they acknowledge that as vaccination protects the mother and the fetus from dangerous illnesses and that malaria (described as "palu," *sumaya*, etc.) is an illness harmful to mother and fetus, when one has been vaccinated (in reality against tetanus); it was therefore against *palu* . . . . In short, if everyone "communicates" often in the form of preventive slogans, no one understands each other. For everyone, the same words can describe different referents.

> Mosquito is the first cause of malaria. After that, there are other causes. It can be foods. If you eat too many fat foods, you can develop malaria. Those who eat too much sugar also fall sick easily. Sugar consumption produces diabetes, but it first starts with malaria. If you want to treat diabetes, you must first cure palu and then completely cure your body of it, otherwise your diabetes will never be cured.

Laypeople's health beliefs result largely from these various combinations of different health "messages" and information conveyed by radios, newspapers, advertisement, movies, and so on. Beyond the single theme of malaria, the question of the effects produced by this compartmentalization of health actions remains to be answered. More generally, this addresses the issue of health policies in developing countries and the capacity of coordination and collaboration at a national or at least at the Health Ministries' level.[4]

Sixth proposal: Collective preventive actions are only one of the elements of complex local policies. Health improvement therefore implies analyzing how medical proposals fit in with specific sociopolitical configurations.

Broadly, the analysis of actors' behaviors regarding the adverse constraints they must take into account is essential if one wishes to link health programs to collective actions from "below" such as drainage of dwellings, collective management of wastes, or any "community" action [54].

A village is never a homogeneous community but corresponds rather to a complex arena of powers and a mosaic of spaces run by different norms. The interior of the home is a

[4] As outlined by a few authors [67] "The essence of a medical anthropological perspective is an appreciation of the complexity of culture and the realization that specific aspects such as health beliefs and behaviours cannot be understood in isolation but need to be looked at in relation to their larger historical, economic, social, political and geographical contexts. Applied medical anthropological research strives to understand the often competing dynamics that shape the various contexts important to diseases such as malaria."

feminine space and its cleanliness is linked to the act of sweeping. But outside, the waste eliminated can become fertilizer and be used by men for their agricultural activities. In the same way, if the streets are public spaces liable to be clean, the borders of mosques are above all religious and must be "pure." They are thus swept by young men or by women in menopause.

Another example is the management of water points, whether it means installing pumps or irrigation channels. This is hardly limited to technical questions. This problem is always an economic and political stake and in particular involves the balances between diverse local powers: Who guarantees the payment of water? Who benefits from these new financial resources? To whom do the installations belong and who must maintain them?

Local political struggles around these questions often involve the use of new equipments. That is why health teams cannot limit themselves to a single technical approach and neglect the modalities of social appropriation of new technologies. One single figure is sufficient proof: In Mali, around 30% of the village hydraulic installations break down after one year of their installation [9,55].

The question of health is therefore included in that of "policies from below" [60] and more generally in the functioning of states capable of ensuring decent living conditions, rules, and a system of social security to its citizens [14].

34.3 ANTHROPOLOGY FOR IMPROVING THE OFFER OF HEALTH CARE

At the end of this brief survey, some simple conclusions stand out. First of all, if one wishes to be understood by those who are addressed, it is essential to know their "expectation horizons": The semantic systems, which receive new information and deal with them [39]. These categories of thinking are neither community nor traditionally based but are notions, which are shared, pluralist, and inscribed in history.

Nor are these "popular conceptions" or insurmountable "barriers." Indeed, they evolve quickly when technical possibilities or an offer of quality health care renders them obsolete. Health education—which consists of giving a social existence to an illness objectified by medical knowledge—cannot be limited to conveying messages but must also apply itself to proposing solutions socially adapted to the contexts encountered: in terms of type of housing, perceptions of nuisances, behavioral norms, and so on. How many health professionals actually sleep under bed nets with their children when it is hot? How many program managers only use bed nets in air-conditioned bedrooms? And, what can be done when health "communicators" do not act in accordance with their messages?

But above all, work is needed to improve the offer of health care and help the principal "variable factor" of these health-related interactions, the health workers, modify their practices. Let us just mention some wide-ranging issues here: improving the patients' reception, understanding that a same illness term can refer to various referents and therefore the need to perfect the diagnostic approach, worrying about the patient's understanding and economic resources, adaptability of recommended preventive measures to the patient's cultural and social background, and so on.

In short, beyond its technical aspects, the medical act is also a social practice and to give only one example, even if a vaccine permitted progress in the eradication of malaria, it would still be necessary to respect the refrigeration chain, ensure a good quality vaccine delivery with committed vaccinators and informed populations.

34.4 THREE OPERATIONAL APPROACHES

Hard pressed by a real urgency and a legitimate desire to be immediately useful, development projects—and particularly health development projects—too often wish to transform worlds they have not taken the time to study or understand [53]. Hence the many mistakes, difficulties, and useless expenditure.

But, action must also be taken without waiting for ideal conditions to be gathered before worrying about prevention and care.

Naturally, one cannot simply regulate health behaviors or human societies. However, three big "basic" principles must be respected in order to elaborate preventive strategies adapted to complex social contexts.

(1) The introduction of an anthropological approach permits us to underline differences between the implementation of essential programs of control of specific pathologies and the elaboration of policies of development having among their objectives and continuous concerns, the prevention of parasitic and infectious pathologies.

The former are "vertical," often linked to occasional financing and come across as answers to precise health problems. In many a case, this intentional limitation is necessary and the focus on a single object confers these projects a degree of effectiveness.

But though useful, these "targeted" actions and these monothematic programs cannot build a durable development.

The duration of secondary benefits (i.e., the fall in the cost of vaccine cover after "commando" operations), the pervert "collateral" effects on other actions or on the daily functioning of services (i.e., "emptying" services for the benefit of the highest bidder, the marginalization of national structures), the difficulties of local reimbursement of actions undertaken (i.e., the impossible national "decentralization" of costly and regional actions), lack of understanding of numerous preventive proposals for populations, must also be evoked and analyzed.

Therefore, it is desirable to complete this kind of project with more regular work of health development that fits into the history of the concerned countries and takes their social

constraints into account (economic possibilities, state of the health system, migration, urbanization, education, agricultural operations, etc.).

The results of these operations "affecting" the link between different sectors of development are difficult to evaluate, and their actions often appear to be "unrealistic" in relation to strict health objectives. And yet, "development projects of dam construction, land reclamation, road construction, and resettlement in Third World countries have probably done more to spread infectious diseases such as trypanosomiasis, schistosomiasis, and malaria than any other single factor" [29].

Nothing is simple about this approach. As we pointed out earlier, the relations between social transformation and health improvement are neither always "positive" nor forcibly linear: Some infectious pathologies are "favored" by development actions especially hydroagricultural, on contrary, improving agricultural production and thus nutrition; others accompany social changes in a more complex way; and still others regress when the conditions of hygiene are improved (trachoma).

Knowing the complexity of these processes has promoted vigilance and an attention to health effects of certain actions of development.

But, this knowledge once in hand, it is important to complete an arrangement of "health vigilance" by actions and multidisciplinary work of health analysis. This would permit a study of the global effects linked to social change, as well as a reflection on "future" "health problems" as much as on the direction of specific pathologies.

> (2) Numerous health programs rightly attempt to positively modify the populations' behaviors. Using various approaches based on local conceptions (*health belief model*), the promotion of ideas or objects (*social marketing*) or local "medias" (*folk media approach*), these actions address and are based on different social groups: children [43], grandparents [3], schools [50].

It must be admitted that in this field of health education, success seems as much linked to the enthusiasm of the animators, to economic availabilities and the vulnerability of pathologies as to a precise method. In addition, the evolution of these actions is difficult and remains to be done [47]. But, because "health messages" associated with others like the press, school, advertising, build a specific public opinion, and a "communicational action" [26] modifying relations to the self, to the other, and to health: It remains necessary to improve the populations understanding about the maintenance of their health and behaviors to be adopted to prevent vulnerability.

This work can only be presented in the form of a constant dialogue, allowing an understanding and evaluation of how a set of empirical practices and popular "knowledge" can coexist and combine with the technical medical knowledge in the current different languages of a society.

Concretely, promoting "basic" education—particularly of women—through school, health training of teachers, policies of alphabetization (in official and vernacular languages) is necessary in the control of infectious pathologies.

> (3) Prevent, inform, heal, and accompany the patient . . . . All these tasks rest on principal actors and health personnel who are the "enabling factors" of the health system.

In developing countries, some experiences of improving relations between health workers and patients have been attempted [34,59]. But this indispensable work should be carried out on a much larger scale and include in the initial training of medical and paramedical students an approach of the various constraints of populations, their way of interpreting the disease, their conceptions of risk and prevention, their modes of evaluating the quality of care.

Including of a social concern in their professional identity is necessary to improve the offer of health and positively transform the behavior of personnel toward their patients.

In fact, beyond their technical competences, health personnel also appear as "go betweens" of modernity. Their training should permit each contact with a health service to be the occasion of a real educational dialogue with the populations.

And it is precisely because of this that the training of these professionals could integrate the various social, linguistic, economic, and affective dimensions of health care, not as a "bonus," which one could eventually add in the form of a few welcoming words during the medical encounter, but, on the contrary, by placing anthropological dimensions at the heart of therapeutic care.

Thus, the question is not of sprinkling a bit of social sciences in the medical curriculum. On the contrary, it is a matter of introducing a truly multidisciplinary approach and showing how the scientific analysis of these social dimensions is essential to ensure not only the patient's respect but also his serious therapeutic care.[5] One cannot improve a health situation without improving the practices of the actors of the health system.

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CHAPTER 35

The Neglected Diseases and Their Economic Determinants

Alvaro Moncayo[1] and Mario Ortiz Yanine[2]

[1]*Centro de Investigaciones en Microbiología y Parasitología Tropical (CIMPAT) Universidad de los Andes, Bogotá, Colombia Formerly, Manager Task Force on Chagas disease World Health Organization/TDR, Geneva, Switzerland*

[2]*Graduate student Parasitology, Centro de Investigaciones en Microbiología y Parasitología Tropical (CIMPAT) Universidad de los Andes, Bogota, Colombia*

35.1 THE "NEGLECTED" DISEASES, CRITERIA FOR CLASSIFICATION

The concept of "neglected/orphan diseases" refers to diseases that in combination affect two-thirds of mankind who live in poverty.

We will consider in this chapter the following diseases that share the criteria enumerated below: African trypanosomiasis (sleeping sickness), American trypanosomiasis (Chagas disease), dengue, leishmaniasis, malaria, schistosomiasis, and tuberculosis. The criteria for the classification of the above diseases as "neglected/orphan diseases" are as given in Table 35.1.

- They affect poor population in poor countries and poor persons in developing and in developed countries alike as it is in the case of tuberculosis.
- They account for a disproportionate amount of the global burden of disease as measured by disease-adjusted life years (DALYs) [34] and by deaths (a DALY is a measure of the economic loss due to the impact of a disease in the productive life of the person and it varies with age).
- They cause high economic loss for the affected countries.
- They do not represent any market appeal for the pharmaceutical industry as the affected populations have low purchasing power.

The above classification of "neglected/orphan diseases" does not mean that they are "*research-neglected diseases*." Consider, for instance, African trypanosomiasis. A recent report published by WHO/TDR features the sophisticated advances in the study of the parasite at the molecular level and the analysis of the parasite genome, always indicating that these studies will revert into the development of new drugs and control tools. But there are no molecules in study to produce new drugs and the current treatment for this disease is based on drugs licensed several years ago (60 years ago for Melarsoprol and 15 years for Eflornithine) [30]. Moreover, a recent resolution adopted by the World Health Assembly in May 2004 requests the strengthening of research, both basic and applied, to treat the disease and to control the vectors [39].

A similar situation can be observed for the lack of new drugs against American trypanosomiasis or Chagas disease. During the last 30 years, research groups in Latin America and elsewhere have produced first class research work in the biochemistry and molecular biology of the parasite *Trypanosoma cruzi*, but no new molecules are in the pipeline for the development of a better treatment [32,33]. The only drug currently available for the treatment of the acute and the indeterminate phases of this disease is Benznidazole, which was licensed more than 50 years ago. Furthermore, the negotiations that started in 2000 between the WHO/TDR and a pharmaceutical company, owner of the patent of an antimycotic with excellent *in vitro* and *in vivo* activity against *T. cruzi*, have collapsed as the patent holder has refused to allow further clinical development of this compound in poor chagasic patients [36].

The examples can be multiplied for other "neglected/orphan diseases," notably malaria, dengue, tuberculosis, and schistosomiasis, to show only that high-quality research in drug/vaccine development is necessary but not sufficient to produce better drugs. The diseases of the poor have been used mainly as models for research by laboratory scientists, and their work has been published in the best scientific journals but has not been translated into useful tools to treat or prevent these diseases.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

TABLE 35.1. Mortality and Burden of Disease Due to Some "Neglected/Orphan Diseases" (Global Estimates for 2002)

Disease	Deaths	DALYs (000)
African trypanosomiasis	48,000	1,525
Chagas disease	15,000	667
Dengue	19,000	616
Leishmaniasis	51,000	2,090
Malaria	1,272,000	46,486
Schistosomiasis	15,000	1,702
Tuberculosis	1,566,000	34,736
Total	2,967,000	87,822

Source: [41].

One noticeable exception is dengue that is receiving increasing attention for the development of better treatments by research groups such as the Singapore-based Novartis Institute for Tropical Diseases where specific antiviral drugs against this disease are being developed [18].

35.2 THE DISEASES [5,8]

35.2.1 African Trypansomiasis

35.2.1.1 Distribution This disease is spread over 36 countries in sub-Saharan Africa (Fig. 35.1).

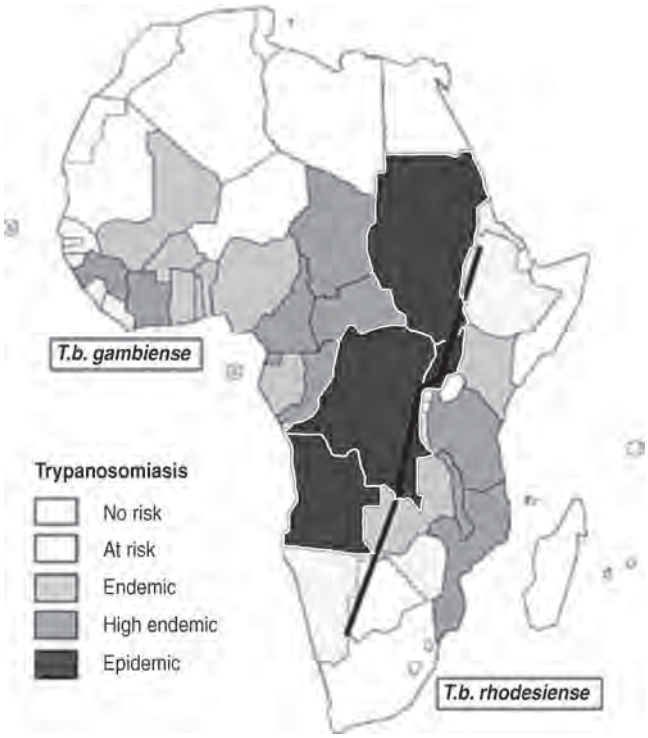

Fig. 35.1. Distribution of sleeping sickness in East and West Africa (www.medicalecology.org/. . . / d_african_trypano.ht).

35.2.1.2 Causative agent Protozoan parasites of the genus *Trypanosoma* enter the bloodstream through the bite of blood-feeding tsetse flies (*Glossina spp.*).

Trypanosoma brucei rhodesiense occurs mainly in East and Southern Africa and *T.b. gambiense* in West and Central Africa. A third subspecies, *T.b. brucei*, is responsible for the disease in cattle but does not infect humans.

35.2.1.3 Transmission It occurs through the bite of bloodsucking male and female tsetse flies that transmit the parasites from one human to another. Cattle and other wild mammals are the reservoirs of the parasites. Tsetse flies can acquire the parasites by feeding on these animals or on an infected person. Inside the human host, trypanosomes multiply and invade most tissues, notably the central nervous system.

35.2.1.4 Diagnosis A sequential approach for diagnosis of *T.b. gambiense* is generally followed: Suspected cases detected by serological methods undergo parasitological diagnosis by investigation of the blood and/or lymph. In case of positive results, examination of cerebrospinal fluid has to follow for stage determination.

T.b. rhodesiense is usually directly detected in the blood.

Immunodiagnostic methods: The most commonly used test for mass screening for *T.b. gambiense* is the card agglutination test for trypanosomiasis (CATT). This test uses a reagent made of stained freeze-dried trypanosomes. The CATT is an agglutination test of high sensitivity and specificity that is easy to perform in the field. Other techniques such as immunofluorescence (IF), indirect hemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA), and molecular techniques such as polymerase chain reaction (PCR) and Southern blot have been described.

Parasitological methods: The body fluids that are most commonly examined for the presence of trypanosomes are blood, lymph node aspirates, and cerebrospinal fluid. Several methods are used for diagnosis in blood such as blood films, concentration methods, and the quantitative buffy coat technique. *In vivo* inoculation of biological material from humans and *in vitro* culture systems can be used for isolating trypanosomes from humans.

35.2.1.5 Symptoms Infection leads to malaise, lassitude, and irregular fevers. Early symptoms which include fever and enlarged lymph nodes and spleen are more severe and acute in *T.b. rhodesiense* infections. Early signs are followed by a range of symptoms including headache, anemia, joint pains, and swollen muscular and connective tissues; advanced symptoms include neurological and endocrine disorders. As the parasites invade the central nervous system, consciousness alteration leads to coma and death.

35.2.2 Malaria

35.2.2.1 Distribution Malaria is found throughout the tropical areas worldwide. *Plasmodium falciparum* prevails in Africa, Southeast Asia, Papua-New Guinea, and Haiti, whereas

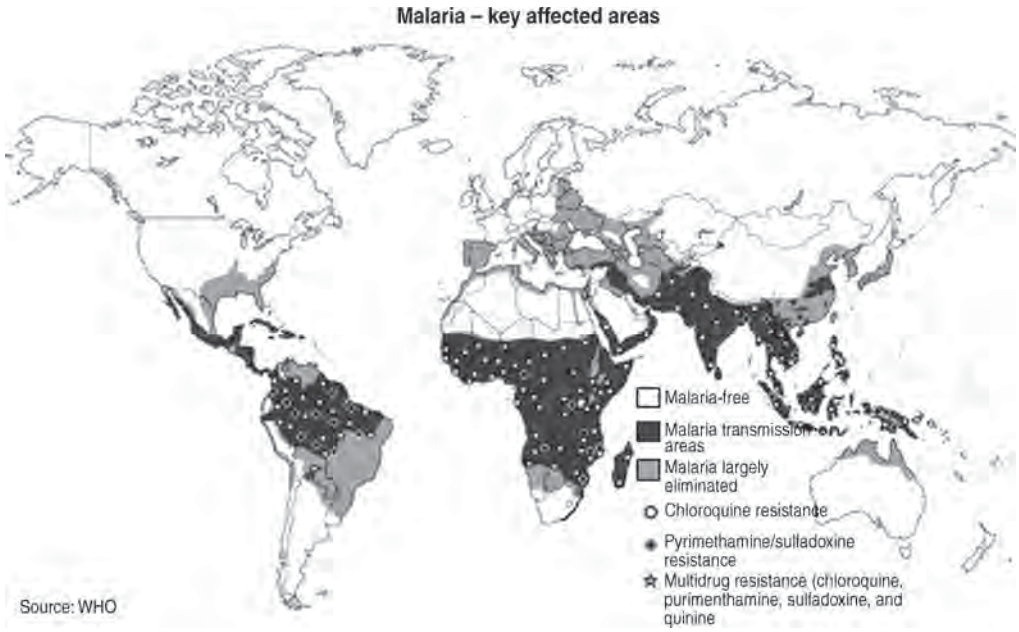

Fig. 35.2. Distribution of malaria worldwide (www.childinfo.org/eddb/Malaria/current.htm).

P. vivax is more common in Central America and parts of South America, North Africa, the Middle East, and the Indian subcontinent. The prevalence of both species is approximately the same in other parts of South America, East Asia, and Oceania. In China and adjacent countries, *P. vivax* strains with long incubation periods and long intervals between relapses may be found (Fig. 35.2).

35.2.2.2 Causative agents Protozoan parasites of the genus *Plasmodium* enter the bloodstream through the bites of blood-feeding anopheline female mosquitoes (*Anopheles spp.*).

The main species that cause human disease are *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*, which are found in many areas but are less common outside Africa.

P. falciparum is the species responsible for the severe, often mortal, cerebral form of malaria in Africa.

35.2.2.3 Transmission Malaria is transmitted by some species of female anopheline mosquitoes that transmit the parasites from one human to another. Malaria transmission does not occur at temperatures below 16°C or above 33°C, and at altitudes over 2000 m above the sea level, because the development of the parasite in the mosquito cannot take place at such conditions. Mosquitoes acquire parasites by feeding on infected humans.

Other ways of transmission are congenital and blood transfusions.

35.2.2.4 Symptoms The most common symptoms include fever, shivering, anemia, and sweating. Some complications and advanced symptoms are present in severe malaria (*P. falciparum*) and include renal failure, pulmonary edema, cerebral malaria, hypoglycemia, and severe anemia that may be lethal.

35.2.2.5 Diagnosis *Blood smears:* Malaria is diagnosed by microscopic examination of the blood. Thick and thin blood films are made on clean glass slides. Two drops of blood are placed at one end of the slide. The thin film is made immediately by placing the smooth leading edge of a second slide in the central drop of blood and while holding the edges of the slide, smearing the blood with a swift and steady sweep along the surface. This film should be dried thoroughly otherwise it may wash away during staining. Giemsa stain yields the best malaria slides. The diagnosis is made by microscopic examination of the slide.

Antigen detection methods: The introduction of simple, rapid, sensitive, highly specific, and affordable dipstick or card tests for the diagnosis of malaria has been a major advance in recent years. These are based on antibody detection of two malaria-specific antigens in blood samples: histidine-rich protein 2 (*pf*HRP2) and parasite lactate dehydrogenase (*pf*LDH).

Current *pf*HRP2 and *pf*LDH tests, based on color reactions, provide a diagnostic sensitivity similar to the blood smear by trained microscopists.

Other techniques: Unlike mature red blood cells, malaria parasites contain DNA and RNA. These can be stained with fluorescent dyes and visualized under ultraviolet light microscopy. DNA probes have also been developed for malaria diagnosis, but their utility outside epidemiological surveys is uncertain.

35.2.3 Leishmaniasis

35.2.3.1 Distribution The leishmaniases are widely distributed around the world. They occur in the intertropical zones of America and Africa, and extend into temperate regions of South America, Southern Europe, and Asia.

The leishmaniases are present in 88 countries in four continents, of which 16 are developed and 72 are developing countries. The different clinical forms of this disease occur in Venezuela, Colombia, Ecuador, Brazil, Mexico, Bolivia, Paraguay, Argentina, Morocco, Tunisia, Algeria, Ethiopia, Kenya, Sudan, Spain, France, Italy, Portugal, Greece, Afghanistan, Iran, Saudi Arabia, Syria, India, Bangladesh, Nepal, and China, among others. The diseases also are prevalent in 13 of the poorest countries of the world (Fig. 35.3).

35.2.3.2 Causative agent Flagellate protozoan parasites of the genus *Leishmania*, these parasites infect numerous mammalian species, including humans, and are transmitted through the infective bite of an insect vector, the phlebotomine sandfly. The genus *Leishmania* includes around 30 different taxa that commonly infect humans. The species *L. donovani* and *L. infantum* are the causative agents of visceral leishmaniasis, a potentially lethal form.

The inoculation of the parasite through the sandfly bite, belonging to the genera *Phlebotomus* and *Lutzomyia*, is the usual way of transmission.

In visceral leishmaniasis a few cases of congenital and of blood transfusion transmission have been reported. Exchange of syringes has been incriminated to explain the high prevalence of *L. infantum*/HIV co-infection in intravenous drug users in Southern Europe.

35.2.3.3 Diagnosis Primarily based on clinical presentation, epidemiological elements, and nonspecific biological parameters, the diagnosis of leishmaniasis is confirmed by direct detection of parasites and/or presence of specific antibodies.

35.2.3.4 Detection methods Collected material can be smeared on to a microscope slide, cultured, fixed for pathological examination, or, more recently, using the PCR technique. Inoculation into laboratory animals is rarely used.

The most appropriate staining method for *Leishmania* detection is the one employing panoptic May Grunwald-Giemsa stain. Direct examination of smears may give a rapid diagnosis if carefully carried out, but has a limited sensitivity below that of culture and particularly below that of new PCR techniques.

Culture has higher sensitivity than direct detection of parasites on smears and is a useful complement to parasitological diagnosis. The classical blood agar NNN medium is the most currently used, but various liquid media are currently available.

Animal inoculation into golden hamsters, one of the most susceptible laboratory animals, is an alternative to *in vitro* cultivation, but is not very practical for clinical purpose.

Molecular diagnosis has been developed during recent years, and can be applied to various types of samples, including bone marrow, spleen, blood, and skin. DNA repetitive sequences are the main probes used. However, PCR tests are not standardized, and few comparative studies have been carried out.

35.2.3.5 Inmmunological diagnosis Various serological tests have been developed to detect circulating specific antibodies for the diagnosis of visceral leishmaniasis.

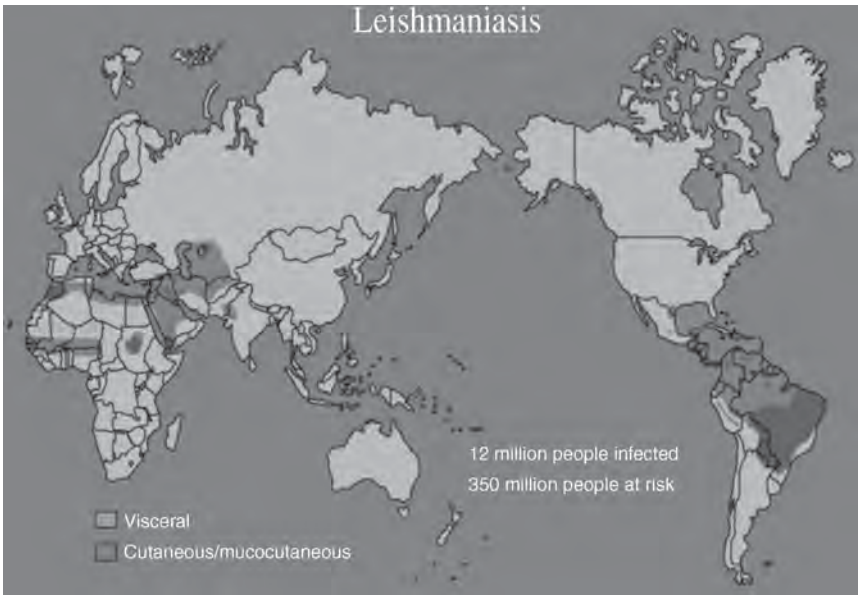

Fig. 35.3. Distribution of visceral and cutaneous/mucocutaneous leishmaniases (www.wehi.edu.au/. . . /handman/leishmaniasis.html).

There are several techniques and they differ in sensitivity and specificity and include indirect fluorescent antibody technique (the most commonly used), immuno-enzymatic techniques, counter-current immuno-electrophoresis, IHA test, and immunoblot. Their use depends on costs and reagent availability.

According to the antigen used, cross-reactions may occur with other infectious diseases such as malaria, trypanosomiases, mycobacterioses, and schistosomiasis. The presence of specific antibodies is not necessarily correlated to an active disease and can reveal a subclinical infection.

The leishmanin skin test measures delayed-type hypersensitivity. This test is usually positive for cutaneous and mucocutaneous leishmaniases. It is always negative during diffuse cutaneous leishmaniasis and it is also negative during acute visceral leishmaniasis in which it usually turns positive several months after clinical cure. The leishmanin test is useful for epidemiological studies.

35.2.3.6 Symptoms In visceral leishmaniasis the most common symptoms are hepatomegaly, splenomgaly, fever with a double daily rise, anemia, internal digestive bleeding manifestations, and diarrhea. This form of leishmaniasis is potentially lethal, specially in children.

In cutaneous leishmaniasis, the most important symptom is the skin lesion without the involvement of the mucosae, and occurs on exposed parts of the body surfaces such as face, hands, and forearms. The mucocutaneous form evolves in two stages: a primary cutaneous lesion, followed by secondary mucosal involvement.

35.2.4 American Trypanosomiasis (Chagas Disease)

35.2.4.1 Distribution Chagas disease is limited to 15 countries of the American continent extending from Argentina to the southern states of the United States. The vector transmission of this disease has been certified in Brazil, Chile, and Uruguay [37] (Fig. 35.4).

35.2.4.2 Causative agent Flagellated protozoan parasites of the species *T. cruzi* is an intracellular parasite in mammals and infects the vertebrate host via the feces of a blood-sucking triatomine insect vector. A characteristic of this parasite is the presence of an organelle composed of mitochondrial DNA – the kinetoplast – which can be seen by light microscopy and is an important diagnostic feature.

35.2.4.3 Transmission The main form of transmission is via the feces of blood-sucking insects belonging to the genera *Rhodnius*, *Triatoma*, and *Panstrongylus*. These insects can acquire the parasites by feeding on animals (opossums, armadillos, bats, dogs, rodents) or on an infected person.

Other forms of transmission have been reported and include blood transfusion, congenital transmission, oral transmission by food contaminated with feces of the vectors, or by ingestion of raw meat of an infected reservoir host.

Fig. 35.4. Initiatives for the elimination of the transmission of Chagas disease (www.who.int/progress/chag_afr/map.htm).

35.2.4.4 Diagnosis Parasitological examination may reveal the presence of trypanosomes in peripheral blood during the acute phase of infection. In some acute cases direct microscopy of unstained wet blood films may be positive.

Methods for detecting scanty trypomastigotes in blood include microscopy of thick blood films, microscopy of the buffy coat layer after hematocrit centrifugation, searching for trypomastigotes in centrifuged serum after blood coagulation, and centrifugation of blood after lysis of red cells with 0.87% ammonium chloride. All these methods may fail to detect trypomastigotes in some acute cases.

The only parasitological techniques that may be effective in the chronic phase are xenodiagnosis and blood culture.

In theory, the detection of parasite DNA by PCR is a reliable alternative to detect intact trypomastigotes in the chronic phase. PCR methods are available but are not yet practical or affordable enough to replace parasitological diagnosis. In addition the PCR method has not yet been validated in population surveys.

Many serological tests have been described and can be applied in the chronic phase of the disease. As there is no effective treatment for this phase of the disease, the specific antibodies are present and can be detected. The most reliable tests are the indirect immunofluorescent antibody test (IFAT) and the ELISA.

35.3.4.5 Symptoms *Acute phase:* This phase can be asymptomatic or subclinical as it does not present specific signs or symptoms, but sometimes there may be an inflammatory lesion, the chagoma, at the portal of entry of the parasite. If the portal of entry is located in the eye, a periophthalmic edema, the Romana's sign, may be present. Other symptoms in the acute phase include fever, myalgia, headache, rash, vomiting, hepatomegaly, splenomegaly, diarrhea, and anorexia.

Chronic phase: Between 20% and 35% of the infected individuals develop, after 15–20 years, the cardiac form of the disease which shows ECG abnormalities, arrhythmias, palpitations, chest pain, edema, syncope, and dyspnea. The chronic cardiac lesions can lead to sudden death.

Six percent of the infected persons develop chronic digestive lesions with abnormalities of the digestive system such as megacolon and megaoesophagus, which require surgical correction.

35.2.5 Dengue

35.2.5.1 Distribution Dengue is a worldwide disease that occurs throughout the tropical and subtropical zones. It is endemic in Southeast Asia, the Pacific, East and West Africa, the Caribbean, and the American continent, and in all these areas the four serotypes of the virus are circulating (Fig. 35.5).

35.2.5.2 Causative agent The dengue virus, a member of the flavivirus group in the family Flaviviridae, is a single-stranded enveloped RNA virus, transmitted through the bites of mosquitoes of the genus *Aedes.* There are four distinct but closely related serotypes of the dengue virus (DEN 1–4).

35.2.5.3 Transmission The dengue virus is transmitted from one human to another by female mosquito bites. These mosquitoes belong to the genus *Aedes* and the most important species involved in the transmission is *A. aegypti*, because of its domestic behavior. Other species of importance are *A. albopictus*, *A. polynesiensis*, and *A. scutellaris*. Transovarian transmission of dengue in the vector has been documented.

35.2.5.4 Diagnosis of dengue fever It is not possible to make a diagnosis of mild dengue or classical dengue from the clinical features alone, as they resemble those of many other diseases. Differential diagnosis includes malaria, leptospirosis, and other viral, bacterial, and rickettsial diseases. The presence of a flushed face, a positive tourniquet test, and leukopenia is helpful in differentiating dengue from other diseases. The clinical diagnosis should be completed by serological tests for antibody detection and virus isolation, or detection of dengue virus antigens using PCR.

35.2.5.5 Diagnosis of dengue hemorrhagic fever (DHF) The World Health Organization established the following criteria for clinical diagnosis of DHF: a continuous high fever of 2–7-day duration; hemorrhagic diathesis; hepatomegaly and shock, together with two laboratory findings – thrombocytopenia ($\leq$ 100,000/mm^3) with concurrent hemoconcentration.

35.2.5.6 Virological diagnosis Etiological diagnosis can be confirmed by serological testing and virus isolation from the blood during the early febrile phase. Antibodies to

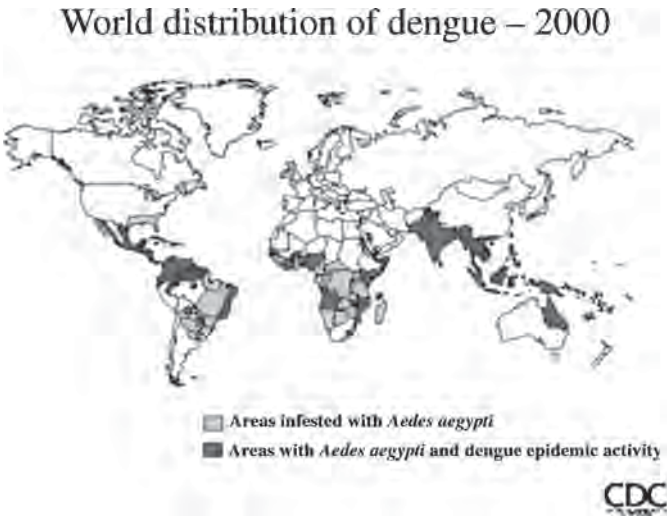

Fig. 35.5. Distribution of *Aedes aegypti*-infested areas and dengue epidemic areas (www.cdc.gov/.../dengue/map-distribution-2000.htm). See color plates.

dengue virus antigens increase rapidly in patients with secondary dengue infection.

Serological diagnosis by detection of anti-dengue IgM and IgG using ELISA is now widely used to document primary and secondary infection. IgM antibody capture (MAC)-ELISA is specific in distinguishing dengue from other flavivirus infections.

The viral RNA can be detected by retro-transcription polymerase chain reaction (RT-PCR) using specific primers designed for the four serotypes and it is possible to distinguish the serotypes using specific primers for each serotype.

35.2.5.7 Symptoms There are two different clinical manifestations:

- The classical dengue fever (DF) with symptoms that include fever, rash, headache, myalgias, joint and bone pains, retro-orbital pain, photophobia, and anorexia.
- The DHF is a severe form of dengue infection which presents diffuse hemorrhages (petechiae and nose, gingival and gastrointestinal tract bleeding) that may progress to fatal shock, the dengue shock syndrome (DSS), as a consequence of plasma leakage from the blood vessels. The liver is often enlarged, soft, and tender, the blood pressure falls below 20 mmHg, and there is a marked hypothermia. Without perfusion treatment the general condition of the patient rapidly deteriorates and death can occur within 12–24 h.

35.2.6 Tuberculosis

35.2.6.1 Distribution Tuberculosis has a worldwide distribution with 2000 million persons infected. The percentage of population infected varies from region to region: in Europe, around 11% of the population is infected – mostly elderly persons – while in tropical countries over half of the population may be infected, with a much higher proportion of younger people. Around 1.5 million cases occurred in sub-Saharan Africa, nearly 3 million in Southeast Asia, and over a quarter of million in Eastern Europe (Fig. 35.6).

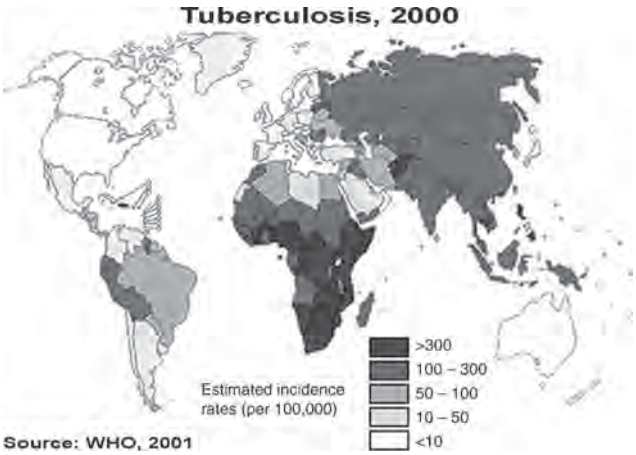

Fig. 35.6. Incidence rates for tuberculosis worldwide (www.travelvacs.ca/. . . / Fact_Sheets12.cfm). See color plates.

35.2.6.2 Causative agent The causative microorganism of tuberculosis, the tubercle bacillus, was isolated and described by Robert Koch in 1882. It was named *Mycobacterium tuberculosis*. A closely related species isolated from cattle, which is also able to cause human tuberculosis, is named *M. bovis*.

Tubercle bacilli are aerobic, nonmotile, nonsporing, often slightly curved rods 2–4 μm in length and 0.3–0.5 μm in diameter. Tubercle bacilli grow slowly on conventional solid culture media, and colonies appear after 2–6 weeks.

35.2.6.3 Transmission Infection of humans with *M. tuberculosis* can occur through various routes:

- Inhalation
- Ingestion
- Inoculation

Congenital transmission is extremely rare. The usual route of infection is via inhalation of small droplets containing bacilli. The usual sources of such infectious particles are other human beings with open pulmonary tuberculosis. A less frequent mode of infection is consumption of milk or food contaminated with *M. bovis*.

35.2.6.4 Diagnosis Diagnosis of tuberculosis is based on a high index of clinical suspicion, and several clinical samples can be subject to laboratory examination. The following are the principal methods used for diagnosis of tuberculosis:

- *Bacteriology:* Identification of *M. tuberculosis* by microscopy and through culture of the organism (gold standard test) from various clinical samples such as sputum, pleural fluid, blood, exudates, bone marrow, lymph node aspirate, and cerebrospinal fluid. Isolation of *M. tuberculosis* in culture from the clinical specimen provides a definitive diagnosis.
- *Imaging techniques:* Including radiology, ultrasound, computed axial tomography (CAT) scanning, magnetic resonance imaging (MRI), and radioisotope scans.
- Tissue biopsy and processing the tissue for histopathology, bacteriology culture, and for molecular identification of mycobacterial DNA.
- Molecular techniques utilizing nucleic acid amplification systems such as PCR or ligase chain reaction (LCR) have been applied to clinical specimens to identify mycobacterial DNA in biological samples.
- Hematology and biochemistry such as erythrocyte sedimentation rate (ESR), C-reactive protein, and liver function tests.

- *Serology:* There have been numerous reports of high antibody levels to a range of antigens of the bacillus, but the low sensitivity and specificity of these serodiagnostic tests limit their diagnostic use.
- *Tuberculin test:* This is usually positive in patients with tuberculosis, although, due to genetic factors, some patients fail to respond. The test does not clearly distinguish between active tuberculosis, past infection by tubercle bacillus, and BCG vaccination.

35.2.6.5 Symptoms The clinical forms can be broadly categorized into pulmonary and extrapulmonary (affecting kidneys, bones, meninges, etc.).

From an epidemiological perspective, the most important clinical form and the most infective one is pulmonary tuberculosis – a chronic disease with respiratory symptoms and a terminal cachectic phase.

The association of tuberculosis and the infection with the human immunodeficiency virus (HIV) is a current and serious public health condition. In Africa, one-third of the population is infected with the tuberculosis bacillus but does not present the clinical forms. The number of persons co-infected with HIV amounted to 17 million in Africa and to 4.5 million in Southeast Asia in 2000. A high proportion of these patients can develop active clinical tuberculosis as the HIV virus weakens the immune system. The number of reported cases of tuberculosis has increased fourfold from the early1990s to 2002. In Eastern Europe the number of patients co-infected with multidrug-resistant tuberculosis and HIV is increasing, and in India there is a proportion of 3% of resistance to the regular treatment [41].

The World Health Organization has launched the directly observed therapy program (DOTS) for tuberculosis treatment. Once patients with infectious tuberculosis (bacilli visible in a sputum smear) have been identified using microscopy diagnosis, health and community workers and trained volunteers observe and record patients taking the full course of the correct dosage of anti-tuberculosis medicines (treatment lasts 6–8 months). The most common anti-tuberculosis drugs are isoniazid, rifampicin, pyrazinamide, streptomycin, and ethambutol.

The drugs were developed more than 40 years ago and there has not been any development of new molecules against this disease since then. The most worrisome perspective at the present is the appearance of resistance displayed by the tuberculosis bacillus which endangers the efficacy of the treatment and control programs launched by the World Health Organization such as Stop TB.

35.2.7 Schistosomiasis

35.2.7.1 Distribution Schistosomiasis is endemic in 76 countries. *Schistosoma mansoni* is found in 55 countries from Arabian peninsula, numerous countries in Africa, and the Indian ocean islands; *S. haematobium* is now endemic in 53 countries in the Middle East, most of the African continent, and some islands in the Indian ocean; *S. japonicum* is found in humans only in mainland China, Indonesia, and the

Fig. 35.7. Distribution and control programs of schistosomiases (www.schisto.org/. . ./global_distribution.htm). See color plates.

Philippines, there is no evidence of recent transmission in Thailand, and schistosomiasis was eradicated in Japan some three decades ago; *S. intercalatum* is endemic in 10 countries in Central and West Africa; *S. mekongi* is endemic in Khong Island, Lao People's Democratic Republic, and some areas of Democratic Kampuchea (Fig. 35.7).

35.2.7.2 Causative agent Worms (Platyhelminthes) of the genus *Schistosoma* are responsible for the disease in humans. The main species found in humans are *S. mansoni*, *S. haematobium*, *S. japonicum*, *S. intercalatum*, and *S. mekongi*. These flukes are peculiar in that they have no second intermediated host in their life cycle and also they mature in the lymphatic and blood vascular system of their definitive host (humans). They are dioecious, instead of monoecious, like the rest of the members of their class (Trematoda). The eggs of these flukes are released in the feces or in the urine (*S. haematobium*) of their definitive host.

35.2.7.3 Transmission It is made through the penetration of the human skin or mucosae by the free-swimming fork-tailed cercariae, when a person is exposed to infested water. After passage through the tissues, lymphatic vessels, and venules, the parasite will develop into a male or female schistosome in the blood vascular system.

35.2.7.4 Diagnosis A definitive diagnosis is made by direct visual demonstration of the eggs of the parasite in body excretions or secretions, mainly stools and urine, or in material from rectal and liver biopsies or surgically removed tissue.

A recent development is the detection of schistosome antigens in serum or urine: the circulating anodic antigen (CAA) or circulating cathodic antigen (CCA). These two glycoproteins associated with the gut of the adult worm are well characterized, they are genus-specific, and their presence indicates active infection in persons with *S. mansoni*, *S. haematobium*, or *S. intercalatum*.

35.2.7.5 Direct diagnosis *Egg counting:* The direct demonstration of eggs represents an enormous advantage over all other diagnostic measures because the specificity of this test is high; egg counts are merely indirect estimates of worm loads because they vary in time and place and with each laboratory technician. For *S. mansoni*, *S. japonicum*, *S. intercalatum*, and *S. mekongi* the eggs are excreted in feces, and for *S. haematobium* the eggs are excreted in the urine.

Rectal biopsy: Is a simple, direct diagnostic technique at the individual clinical level; this kind of biopsy provides an effective way to visualizing eggs.

35.2.7.6 Indirect diagnosis *Chemical reagent strips:* This type of diagnostic is used frequently in *S. haematobium* infections; these strips detect red blood cells or hemoglobin in the urine, a very common symptom in *S. haematobium* infections.

*Immunodiagnosis: S*erodiagnostic techniques are used for the detection of either species-specific antibodies or genus-specific antigens.

Antibodies to adult worm, schistosomular, cercarial, or egg antigens are detected by a multiplicity of procedures such as ELISA, radioimmunoassay (RIA), IFAT, gel precipitation techniques (GPT), IHA, and latex agglutination (LAT). Their common disadvantage is that they all point to past exposure to mammalian or, in rare cases, avian schistosomes. Another disadvantage is the absence of globally agreed criteria of performance and standards.

In contrast, advances have been made in antigen detection due to improvements in the production of monoclonal antibodies against CAA and CCA.

35.2.7.7 Symptoms The symptoms are consequent to the general dissemination of the eggs of the schistosomes in the different organs of the body. The main symptoms in the acute phase are fever, general malaise, and diarrhea (Katayama's syndrome). The chronic disease is progressive and there are several clinical pictures according to the different affected body systems. Ascites is a typical manifestation of this disease due to the obstruction of the lymphatic vessels in the abdominal cavity. Cirrhosis is an important lesion in the liver that leads to the fibrosis of the portal vein that produces esophagus varices and hematemesis, which may be fatal. A serious clinical manifestation is the pulmonary obstruction which produces dyspnea and cyanosis. The co-infection with hepatitis B and C viruses increases the risk of hepatic carcinoma.

In the case of *S. japonicum* the eggs of the parasite reach the brain with the consequent symptomatology and in the case of infection with *S. haematobium* the affected organs belong to the genital and urinary systems producing papillomatosis, hematuria, and vesical cancer.

35.3 THE "NEGLECTED" DISEASES BURDEN

As indicated in Table 35.1, the burden of disease that is estimated by the economic value of the healthy life years of an individual amount to 87,822 DALYs or 14.4% of the total world DALYs and to 2,967,000 deaths or 27.2% of the global deaths due to infectious and parasitic diseases [41].

Some "neglected/orphan" diseases such as guinea worm, leprosy, lymphatic filariasis, Chagas disease, and onchocerciasis have experienced important reductions in the incidence of cases and hence in the burden of disease, due to the Government financial commitment in the affected countries during the 1970s–1980s and early 1990s, when the traditional government programs were still operative and had enough government budget [22].

35.4 THE ECONOMIC SITUATION AND TRENDS IN THE AFFECTED COUNTRIES AND REGIONS

In the present world economic order, poverty is the rule, and wealth and well being are the exceptions, thus making inequity one structural characteristic of the social and economic world situation. The 225 biggest fortunes of the world amount to more than US$ 1000 billion, which represent the income of 47% of the poorer world population or 2500 million persons. Furthermore, whereas the global production of food can cover 110% of the world needs, 30 million of persons per year die of hunger and 800 million are undernourished [26].

35.4.1 Latin America, 1990–2003

"The Latin American countries have not been exempted of the impact of globalization, and the standards of living of millions of Latin Americans is today lower than at the beginning of the 1970s" [3].

In the past decade, the economic situation of most populations of Latin America has deteriorated as shown by several economic indicators. The unemployment in the economically active population, for example, has increased from 7.7% in 1994 to 10.6% in 2002. Also, the income concentration in the richest households in the continent indicates the increase in poverty: 10% of the urban richest households have an income that is up to 40-fold of that in the poorest ones [6].

In addition the gap in the share of the income has widened to reach the disproportionate situation in which one-tenth of the richest population earns 40% of the total income, whereas one-tenth of the poorest population earns 1% of the total income [24].

Furthermore, between 1994 and 2002, the gross national product (GNP) fell from 5.2% to –0.4% and the GNP per capita decreased from 3.4% to –1.9% [4,6].

The external debt of the Latin American countries amounts to US$ 744 billion, which means that each inhabitant of the continent has to pay roughly US$ 2000 while 47% of them live below the poverty line with US$ 1 per day. The net transfer of monetary resources has passed from US$ 11 billion that entered the economies in 1994 to US$ 40 billion that have *come out* from the countries in 2002 as payments of debt interests to the international creditors [7].

The Gini coefficient that measures the distribution of wealth in a population and varies from 0 to 1 (if there was a minimum concentration of wealth, the coefficient would be close to 0 and if there was a maximum concentration of wealth it would be close to 1) has increased in the continent during the 1990s, indicating a high concentration of wealth and hence an increase of poverty. This indicator shows a tendency to increase in most of the countries between 1990 and 1998. In Brazil it has increased from 0.57 to 0.59, in Chile from 0.55 to 0.57, in Honduras from 0.54 to 0.56, in Panama from 0.56 to 0.58, in Peru from 0.47 to 0.51, in Venezuela from 0.46 to 0.59, and in Uruguay from 0.40 to 0.41 [29].

There are differences in the level of poverty among areas and regions in the countries. The lowest extreme poverty is found in the urban populations as compared to the rural ones where the worst negative economic impact is suffered.

Also, it should be mentioned that globally over 17% of the population in the developed countries is poor and that 30% of the people in developing countries – some 1300 million persons – survives with US$ 1 per person per day. In Europe there are 165 million persons who live below the poverty threshold, with 4.4 Euros per day.

This gloomy perspective – consequence of the forced adoption by the Governments of Latin America of a neoliberal economic model promoted by the International Monetary Fund (IMF) and the World Bank (WB) – has led to the President of the World Bank to declare that the "*consensus of Washington is dead*" thus indicating the need to articulate an integral development and a vigorous social policy aiming at an "*economy with a human face*." [2].

35.4.2 Africa, 1985–2003

Ten out of 14 West-African countries of the economic area of the Franc (CFA), that is, Benin, Burkina Faso, Ivory Coast, Mali, Niger, Senegal, Togo, Cameroon, Central African Republic, Congo, Gabon, Guinea Bissau, Equatorial Guinea, and Chad, are among the less advanced countries of the world and 90% of their population survives with less than US$ 1 per day.

In 1994 these countries were forced to a brutal devaluation of their common currency, the CFA franc, with the consequent reduction of purchasing power due to the restrictive measures imposed by the IMF and the World Bank. The exports from these countries toward Europe between 1985 and 1993 were reduced and caused a negative balance in view of the low prices of the raw materials, the main products for export from these countries. In addition, the imports of products from the developed countries contributed to negative commercial balance and to the degradation of 35% in terms of interchange between 1981 and 1997.

These circumstances weakened the African economies and increased the dependency of these countries in the exportation of primary agricultural and raw mining products. Still in 2000, these items accounted for 70% of the exports.

In sub-Saharan Africa, the annual economic growth has further fallen from 2% in 1995 to 0% in 2001 [24]. The yearly payments of the service of the external debt amounts to US$15,000, which is four times more than the total budget allotted to the continent for education and health. The inevitable consequence has been the decreasing investment of the Governments in health and education programs [14].

All the above countries are endemic for malaria and African trypanosomiasis. Ninety percent of the world deaths due to malaria occurs in Africa and, at the present, there is an increase in the number of new cases of African sleeping sickness since 1995, after a period of more than 20 years of continuous decrease in the incidence of this disease, consequent to the regular control operations of the national programs.

TABLE 35.2. Mortality and Burden of Disease by Selected Diseases, Africa 2002

Disease	Deaths (000)	% World	DALYS (000)	% World
Malaria	1,136	90.0	40,855	88.0
African tripanosomiasis	48	100.0	1,525	100.0
Tuberculosis	348	23.0	9,266	27.0

Source: [41].

Moreover, as shown in Table 35.2, the burden of disease due to tuberculosis in Africa represent 27% of the worldwide burden, and the continent suffers 88% of the disease burden due to malaria in the world.

35.4.3 Southeast Asia, 1990–2001

The economic growth in most of the countries of the subcontinent has been constant with an average for the region of 7.6% (1980–1990), 10.3% (1990–1995), and 7.2% (1998–1999), the lowest figure consequent to the economic crisis of 1997–1998 in Indonesia, Thailand, Malaysia, and Korea [28].

However, the proportion of population living below the poverty line (less than US$ 1 per day) ranges from 46.1% in Lao Popular Democratic Republic and 44.2% in India, to 11.3% in Indonesia (1993–1996).

The Gini coefficient (if there was a minimum concentration of wealth, the coefficient would be close to 0 and if there was a maximum concentration of wealth, it would be close to 1) is 0.3 in Lao PDR, 0.36 in Vietnam, 0.4 in Thailand, 0.36 in Indonesia, and 0.48 in Malaysia.

The proportion of population with access to sanitation in urban areas varies in different countries and is 100% in Malaysia, 98% in Thailand, 88% in India, 70% in Lao PDR, and 43% in Vietnam.

Life expectancy ranges from 68 years in Vietnam to 71 years in Malaysia. The percentage of government expenditure in social services including education, health, social security, and housing in 1998 spans from 42.50% in Malaysia, 38.3 in Thailand, 30.5% in Vietnam, and 26.2% in Indonesia.

The under 5 mortality rates in the region varies between 96 × 1000 live births in India and 12 × 1000 in Malaysia. The percentage of child malnutrition under age 5 ranges from 46.7% in India and 40% in Lao PDR to 20% in Malaysia [35].

The countries in the region are endemic for malaria, dengue, leishmaniasis, tuberculosis, and lymphatic filariasis. Table 35.3 features the mortality and the burden of disease due to the above-mentioned diseases: 63% of the world deaths caused by dengue, 70% of those due to leishmaniasis, and 38% of those due to tuberculosis are originated in Southeast Asia. Likewise, 60% of the global disease burden by dengue, 24% of the burden by leishmaniasis, and 40% of the burden by tuberculosis are originated in this subcontinent.

35.5 ECONOMIC BARRIERS FOR DEVELOPMENT OF DRUGS, VACCINES AND VECTOR CONTROL TOOLS AGAINST THE "NEGLECTED DISEASES"

In the current global situation where there is a clear inequity in the distribution of political and economical power between developed and developing countries, the implicit question arises whether research and scientific innovation should be centralized only in those countries that can afford it. In our opinion the answer to this question is *no*. Science and research are the products of the free exercise of human intelligence and are not *per se* bound to or determined by economic affluence alone. The political consequences could be disastrous for the advanced and the less developed countries alike.

When the most prestigious scientific and biomedical journals report on the latest scientific breakthroughs, the justification always appears to explain that this or that advancement of basic research will soon be applied to cure or prevent related diseases.

There is no need to justify research in biomedical sciences anticipating its possible therapeutic or preventive application. Basic research stands by itself as the free exercise of human intelligence. It is necessary for the advancement of knowledge and science. This, however, does not imply that science and research are human activities that occur in isolation from political and economical circumstances or that they are neutral in themselves.

The technological and economic processes that allow for the development of products (drugs, vaccines, diagnostic tests, etc.) derived from the new knowledge are to be seen as responsible of the barriers that block the flow from basic

TABLE 35.3. Mortality and Burden of Disease by Selected Diseases, Southeast Asia 2002

Disease	Deaths (000)	% World	DALYS (000)	% World
Malaria	65	5.1	5,052	11.0
Dengue	12	63.1	381	62.0
Leishmaniasis	36	70.6	1,358	65.0
Tuberculosis	599	38.2	13,931	40.1

Source: [41].

research to product development. In other words, there is a contradiction between science and technology that prevents the translation of basic research into useful tools to cure or prevent the diseases of the poor [19].

The above applies in particular to some of the so-called "neglected/orphan diseases" such as malaria, tuberculosis, Chagas disease, and African trypanosomiasis that are not "*research-neglected diseases*" as discussed earlier in Section 35.1.

The development of products for the "neglected/orphan diseases" that account for 14.4% of the disease burden due to infectious and parasitic diseases and are prevalent in the developing countries has no commercial appeal for the pharmaceutical industry. As it was put bluntly in a recent issue of a known magazine: "Pharmaceutical companies have little incentive to develop treatments for diseases that particularly affect the poor" [1].

But there are many examples of high-quality scientific research that are currently carried out in developing countries and are aimed at the solution of the public health problems that directly affect those countries. Research on dengue in Cuba and Thailand or the studies on malaria vaccines in Colombia and the sequencing of *T. cruzi* genome in Brazil [10] are good examples to be cited that are widely acknowledged.

The real barriers that prevent the translation of research work into the development of better drugs, vaccines, and so on are of an *economic nature* as the poor patients and countries affected by these diseases are not attractive markets that justify any investment from the transnational pharmaceutical industry. These limitations to translate basic research findings into useful products are economic as the pharmaceutical industry is technology-based and essentially profit-driven. So, these transnational laboratories are *neglecting* the development of drugs for treatment of the diseases that do not represent immediate or short-term profit.

A review of the mechanisms and processes for the lack of drug development against the diseases of the poor can illustrate the point. Figures from a recent article on the development, between 1974 and 1999, of new molecules with therapeutic activity indicate a dramatic imbalance between the numbers of those new drugs for the treatment of diseases more frequent in the developed world as compared to those conditions more frequent in the poor countries. The proportion of new drugs follows closely the world market share of these new compounds.

While there have been 211 new drugs (15.1% of the total) for the treatment of disorders of the nervous system – corresponding to 15% of the world sales – and 179 new drugs (12.8% of the total) for the treatment of cardiovascular diseases, with 19.8% of the world sales, there have been 26 new antiretrovirals (1.9% of the total) and only 13 (0.9%) new therapeutic molecules developed against all tropical diseases that represent a meager 0.2% of the world sales [31]. This is an unfair and unethical situation in all respects.

The cost of the development of a new drug is estimated in the order of US$ 500–700 million, an amount that is higher than the total national budget, in several decades, allotted for all research in an advanced developing country. But there is also a manipulation of the costs of a new drug. Recent figures indicate that the big pharmaceutical industries expend twice as much in marketing and publicity than that in research and development. In the year 2000 a multinational laboratory spent US$ 3800 million in marketing, exactly twice as much as the US$ 1930 million that were invested by the same laboratory in that year in research and development [9].

Furthermore, the enforcement by the World Trade Organization (WTO) of the Trade Related Intellectual Property Rights (TRIPS) agreement, disregarding the safeguards that are explicit in the text, and in spite of the Doha decisions agreed upon in 2001, to allow that some advanced developing countries could produce less expensive drugs, has been limited only to tuberculosis, malaria, and HIV/AIDS.

There are some examples of country efforts aimed at correcting this picture at the risk of retaliation by the developed countries in the tribunals of the WTO; for instance, the effect of the introduction of generic drugs on the reduction of prices of combination treatment against AIDS. Between September 2000 and August 2001, the price of the combination therapy with branded drugs dropped by 97% after the introduction in the market of less expensive generic products by an Indian company [11].

The imposition of unfair conditions in the signature of bilateral free trade agreements (FTAs) between the United States and the Central American and Andean countries (Colombia, Ecuador, and Peru), Chile, and African countries like Morocco will lead to disastrous consequences in the area of development of generic less expensive drugs, because these and other developing countries have been forced to accept conditions that favor the transnational pharmaceutical industry. One example of this unfair play in these FTAs is the prolongation by the developing country of the drug patents for an extra period of between 10 and 30 years beyond the limits of validity of the patent which obviously affect the local production of generic drugs [12].

Using this tactic, the developing countries are forced to accept unfair conditions preventing them to apply the safeguards established in the Doha Declaration of the WTO concerning the TRIPS agreement and Public Health [38,43].

In a recent review on the impact in public health of the implementation of the TRIPS agreement in Latin America and the Caribbean, it was shown that the countries have not used the provisions framed in the agreement and, therefore, have not ensured the access of the population to generic drugs. This situation can further deteriorate if more restrictive agreements such as the FTAs come in force [44].

In conclusion, there is a contradiction between the noble purposes of research and the economic reality of developing countries as the determinants of the global economy are not conductive to local production of less expensive drugs, vaccines, and insecticides in the developing countries where they are desperately needed. It is not the intrinsic value of research that prevents the development of useful tools to help alleviate

the heavy disease burden in the developing world, but the many barriers imposed by an economic world model that generates poverty in the developing countries consequent to the undue accumulation of wealth in a few countries in the developed world.

35.6 FUTURE PERSPECTIVES

The Drugs for Neglected Diseases initiative (DNDi) launched recently by Médecins Sans Frontières [20] proposes the direct involvement of the research centers and the local pharmaceutical industry in the endemic countries. They have to be subsidized by the local governments in private–public ventures.

In this respect, there is a proposal for the establishment of the Latin American Network for drug development against "neglected/orphan diseases." The network will involve government agencies such as FIOCRUZ/Farmanguinhos in Brazil and the local pharmaceutical industry from the advanced Latin American countries including Brazil, Argentina, Colombia, and so on.

Also, a Latin American Network for Vector Control (RELCOV for the Spanish acronym) was recently established [27]. The network involves government control programs, research centers, and the national insecticide industries, and was founded to optimize vector control activities in Latin America and to prioritize research, development, and local production of insecticides for use in public health.

For the transnational pharmaceutical industry the development of insecticides for public health is no longer a priority as all efforts will be centered in the development and production of pesticides for use in agriculture, as it was clearly stated by the representative of the transnational industry at a recent meeting of the World Health Organization Pesticide Evaluation Scheme (WHOPES) held in WHO Headquarters in Geneva, Switzerland, in June 2004 [40].

The perspective is gloomy in all respects. No new drugs are to be developed and no new insecticides are in view for the treatment and the prevention of the "neglected/orphan diseases." Both situations are determined by economic factors that depend upon the current unfair and asymmetric economic order where there is an undue accumulation of wealth in a few developed countries at the expense of the poverty and suffering of most of mankind.

However, it is time that the national pharmaceutical industry in the endemic countries work with the research centers and with regional and/or national financial agencies to fill this gap in the development of drugs and insecticides against the "neglected/orphan" diseases left by the transnational pharmaceutical industry. It seems that this can be the case of future political and economic initiatives in this respect, as demonstrated by the recent alliance between Brazil, Nigeria, China, Ukraine, and Russia, launched at the 15a International Conference on AIDS held in Bangkok in July 2004, to develop generic antiretrovirals and diagnostic tests [15].

A private pharmaceutical company, Chemotécnica, from Buenos Aires, has developed a fumigant canister for release of insecticide fumes that has proven very useful for control of triatomine insects, the vectors of Chagas disease, and for the control of *A. aegypti*, the vector of dengue fever [17,25].

In a recent report issued by the World Health Organization [45] it is recognized that "pharmaceutical research and development are based on a market-driven incentive system relying primarily on patents and protected pricing as a prime financing mechanism." Hence, those diseases, such as the "neglected/orphan diseases" discussed above, are left aside as they affect groups of poor populations with a low purchasing power. The same report advocates for a greater involvement of private–public partnerships and other not-for-profit drug development initiatives to develop new molecules for the treatment of the above diseases, and specifically mentions tuberculosis, malaria, trypanosomiases, and leishmaniasis.

Other initiatives such as the Health Innovation Networks discussed in a recent paper [23] have been established in developing countries as a response to the gross inequities in disease burden between rich and poor countries. These initiatives focus on a broader concept of health innovation that include drug, vaccine, and vector control research and development ventures.

Since the mid-1990s, Health Innovation Networks have been established and have a good record in research and development. These include the FIOCRUZ-WHO/TDR Parasite Genome Network established in 1994, the Developing Country Vaccine Manufacturer's Network operative since 2000 which involves public and private funds, the South–South Initiative for Tropical Diseases founded in 1991, and the Technological Network on HIV/AIDS that supports research and production of antiretroviral drugs and new formulations.

There are several points in this respect that deserve further analysis for the development of public funding of research and manufacturing policies:

- The "developing countries" constitute a group of countries with varying degrees of scientific innovative capabilities and manufacturing infrastructure.
- Eleven developing countries, that is, Argentina, Brazil, China, Hong Kong, India, Malaysia, Mexico, Singapore, South Korea, South Africa, and Taiwan have made major efforts in patentable innovations during the 1990s. The number of patents granted by the US Patent and Trade Mark Office to these countries has increased by 75%, from 277 in 1990 to 1077 in 2003 [16].
- In many developing countries health research and manufacturing facilities of public health products are funded by the public sector. Examples include FIOCRUZ/Biomanguinhos in Brazil, the Serum Institute of India, the Finlay Institute in Cuba, and so on.
- Manufacturing and distribution of low-cost products in developing countries to make them affordable for low-income groups.

- The public sector in many advanced developing manufacture products that are relevant to public health interventions and are well accepted by the communities in need.

There is some hope in the implementation of public–private partnerships (PPP) for the development of drugs for some neglected diseases as reported recently. The optimization of the respective advantages of the private pharmaceutical industry in the areas of basic research and discovery of new leads combined with the sponsorship by the public sector of the endemic countries of the preclinical and clinical studies has resulted in a shortening of the time needed for the development of a new drug with substantial cost savings [21].

As a final note we wish to quote the words of Bill Gates in his address to the World Health Assembly in May 2005, that summarize very well the current world economic forces responsible for the ill health of the poor: "To find new discoveries and deliver them, we need to make political and market forces work better for the world's poorest people. The market works well in driving the private sector to conduct research and deliver interventions, but only for people who can pay." [42].

The inequities in health between rich and poor countries recognize an economic determinant of paramount importance: the prevalent paradigm of open market economies that shapes the current world economic order is the cause of these inequities, and the public sector in the developing countries has to exercise its regulatory function to help solving this unfair situation.

ABBREVIATIONS AND ACRONYMS

AIDS:	Acquired immunodeficiency syndrome
ALANAM:	Asociación Latinoamericana de Academias de Medicina
BCG:	Bacile de Calmet-Guérin. Antigen for Tuberculosis vaccine
CAA:	Circulating anodic antigen
CCA:	Circulating cathodic antigen
CAT:	Computed axial tomography
CATT:	Card agglutination test for trypanosomiasis
CEPAL:	Comisión Económica para América Latina (UN Economic Commission for Latin América)
CIMPAT:	Centro de Microbiología y Parasitología Tropical, Bogotá
DALYS:	Disease-adjusted life years lost
DEN 1–4:	Dengue virus serotypes 1–4
DF:	Dengue fever
DHF:	Dengue hemorrhagic fever
DSS:	Dengue shock syndrome
DNA:	Dexorribonucleic acid
DNDi:	Drugs for neglected diseases initiative
DOTS:	Directly observed therapy for tuberculosis
ELISA:	Enzyme-linked immunosorbent assay
ESR:	Erythrocyte sedimentation rate
FIOCRUZ:	Fundaçao Instituto Oswaldo Cruz, Brazil
FTAs:	Free trade agreements
HIV:	Human immunodeficiency virus
IFAT:	Immunofluorescent antibody test
IMF:	International Monetary Fund
IHA:	Indirect hemmagglutination test
IgM:	Immunoglobulin M
IgG:	Immunoglobulin G
LAT:	Latex agglutination technique
LCR:	Ligase chain reaction
MAC-ELISA:	IgM antibody capture-ELISA
MSF:	Médecins sans frontières
MRI:	Magnetic resonance imaging
PCR:	Polymerase chain reaction
RELCOV:	Red Latinoamericana de Control de Vectores
RNA:	Ribonucleic acid
RIA:	Radioimmuno assay
RT-PCR:	Retro-trascription polymerase chain reaction
TRIPS:	Trade agreements related to intellectual property
TDR:	UNICEF/UNDP/WORLD BANK/WHO Special Program for Research and Training in Tropical Diseases
WHA:	World Health Assembly
WHO:	World Health Organization
WHOPES:	World Health Organization Pesticide Evaluation Scheme
WTO:	World Trade Organization

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CHAPTER 36

The Challenge of Bioterrorism

Stephen A. Morse

Centers for Disease Control and Prevention, Atlanta, Georgia, US

36.1 INTRODUCTION

A number of events over the last decade have served to focus attention on the threat of terrorism and the use of biological, chemical, or nuclear weapons against military and civilian populations for the purpose of causing illness or death. In addition, it has been increasingly recognized that agricultural animals and plants also present a vulnerable target to terrorists [56,114]. Perhaps, most significantly, the threat of terrorism has attracted the attention of policy makers in all levels of government in the United States and, more recently, in a number of foreign countries. However, these policy makers and analysts have differed in their assessment of the threat of bioterrorism. Before 2001, many authorities believed that the threat of bioterrorism was growing, particularly from non-state sponsored groups [22]. Some contended that it was only a matter of time before a terrorist used biological agents to cause mass casualties, while others argued that the historical record provided no basis for concern. Moreover, some even questioned the wisdom of funding preparedness efforts [32]. However, the situation changed in October 2001 when an individual or individuals sent spores of *Bacillus anthracis* (Fig. 36.1) to media companies in New York City and Boca Raton, Florida, resulting in five deaths [63], considerable panic throughout the United States [40,79] and other countries [86], and raised the awareness of our vulnerability.

Bioterrorism presents many challenges, particularly when compared to chemical, radiological, or nuclear terrorism. These challenges reflect the dual-use nature of many technologies that can be used for either beneficial purposes or bioterrorism; intentional threats that can coexist with similar and naturally occurring threats; the complexity of the interaction of the threat with the environment, the human immune system, the society/social structure; and rapidly accelerating advances in biotechnology. Preparing for and responding to bioterrorism has brought together disparate disciplines with different backgrounds and cultures that are still learning to work together [46]. Among these are intelligence agencies, public health and medical professionals, law enforcement officers, scientists, and the commercial sector. This article will examine many of these issues and challenges.

36.2 DEFINITIONS

For the purposes of this article, the working definition of a *biological agent* is a microorganism (or a toxin derived from it), which causes disease in man, plants, or animals, or causes deterioration of material [96]. The use of such biological agents is often classified by the manner in which they are used. For example, *biological warfare* (synonymous with biowarfare) has been defined as a specialized type of warfare conducted by a government against a target (e.g., human, agriculture, or infrastructure) [23]; *bioterrorism* (synonymous with biological terrorism) has been defined as the threat or use of biological agents against the aforementioned targets by individuals or groups motivated by political, religious, ecological, or other ideological objectives [23]; and a *biocrime* has been defined as the use of biological agents for murder, extortion, or revenge [23]. Although this article focuses on bioterrorism, much of the discussion also applies to biological warfare or biocrimes – the consequences of which are likely to be similar whether conducted by a nation-state during a formally declared war or by a hostile actor outside of war.

36.3 THREAT AGENTS

A comprehensive literature review identified 1415 species of infectious organisms recognized to be pathogenic for humans [101]; many more are capable of causing infection in animals or plants. Realistically, only a few pose serious problems or

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

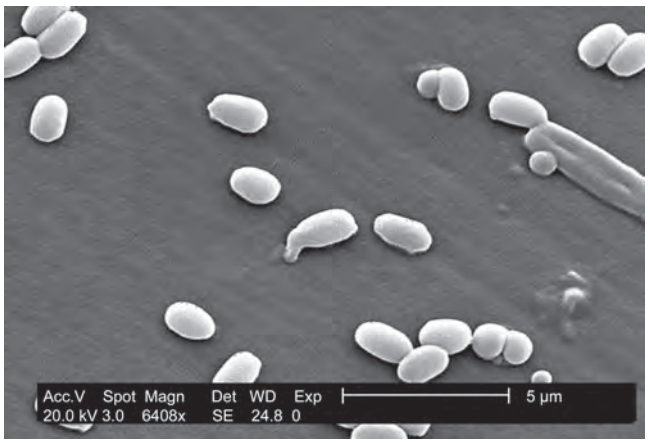

Fig. 36.1. An electron micrograph of spores from the Ames strain of *Bacillus anthracis*. A similar strain was used to produce the spores that were sent through the US mail to media companies in the fall of 2001. These spores are resistant to many environmental conditions enabling the bacteria to survive in a dormant state. When inhaled, these spores can reach the alveoli of the lungs where they are engulfed by pulmonary macrophages [provided courtesy of Laura Rose, Ph.D., CDC].

are capable of affecting human, animal, or plant health on a large scale. Various criteria such as ease of production and dissemination, transmissibility, environmental stability, and morbidity and mortality rates have been used to identify the biological agents that could be used as weapons against humans, animals, or plants. For example, the US military has formally assessed multiple agents for their strategic usefulness on the battlefield [107]. In addition, the Working Group on Civilian Biodefense, using an expert panel consensus-based process, has identified several biological agents as potential high-impact agents against civilian populations [8, 37, 50, 58, 59]. Many of these lists of potential biological threat agents are based on unclassified documents (Table 36.1), which have included: (i) the Australia Group Core List for Biological Agents for Export Control [10]; (ii) the Biological Weapons Conventions List [4]; and (iii) the US Department of Agriculture (USDA) [5] and US Department of Health and Human Services (HHS) Select Agent Lists [51].

As part of an effort to focus public health preparedness efforts, the US Centers for Disease Control and Prevention (CDC) also developed a prioritized list of biological agents [28] taking into consideration criteria such as high morbidity and mortality; potential for person-to-person transmission, directly or by vector; low infectious dose and high infectivity by aerosol, with a commensurate ability to cause large outbreaks; ability to contaminate food and water supplies; lack of a specific diagnostic test and/or effective treatment; lack of a safe and effective vaccine; potential to cause anxiety in the public and in health care workers; and potential for weaponization. The final category assignments (A, B, or C) of agents for public health preparedness efforts were based on an overall evaluation of the ratings the agents received in the areas of (a) public health impact based on illness and death; (b) delivery potential to large populations based on stability of the agent, ability to mass produce and distribute a virulent agent, and potential for person-to-person transmission of the agent; (c) public perception as related to public fear and potential civil disruption; and (d) special public health preparedness needs based on stockpile requirements, enhanced surveillance, or diagnostic needs [94]. Agents placed in Category A were considered to have the greatest potential for adverse public health impact with mass casualties, and most required broad-based public health preparedness efforts. Category A agents also have a moderate to high potential for large-scale dissemination or a heightened general public awareness [83] that could cause mass public fear and civil disruption. Most of the agents placed in Category B also have some potential for large-scale dissemination with resultant illness, but generally cause less illness and death and, therefore, would be expected to have lower medical and public health impact. These agents also had lowered general public awareness than Category A agents and required fewer special public health preparedness efforts. Agents in this category required some improvement in public health and medical awareness, surveillance, or laboratory diagnostic capabilities. Several biological agents that could be widely disseminated in food or water were included in this category.

Biological agents that were not believed to present a high bioterrorism risk to public health but which could emerge as future threats, as scientific understanding of these agents improved, were placed in Category C. New agents continue to emerge as significant public health threats. Some, such as the novel coronavirus associated with severe acute respiratory syndrome, have properties (e.g., transmission via the respiratory route, high mortality in some age groups, and lack of treatment) similar to agents that have been weaponized [39, 55, 69]. The emergence of new public health threats coupled with improvements in preparedness necessitates the periodic reevaluation of the threat lists.

In an effort to provide a quantitative assessment, Casadevall and Pirofsky [24] developed the concept of the "weapon potential" (WP) of a microbe, which was defined as the suitability of a microbe as a biological weapon. The WP of a microbe could be mathematically expressed as:

$$\mathrm{WP} = [V_{\mathrm{BW}} SC/T] \times XD$$

where V_{BW} = the virulence of a biological weapon, which was derived from F_{SI}/I; F_{SI} is the fraction of symptomatic infections for a given inoculum (I); S = stability of microbe when released; C = host-to-host communicability; T = time; X = terror modifier based on judgment that the agent could cause panic and social disruption; D = deliverability of the agent (a function of the technical capabilities of the user and the biological characteristics of the microbe).

Unfortunately, the information to calculate the WP for the overwhelming majority of pathogenic microbes is lacking. Moreover, the calculation of WP for the well-studied agents,

TABLE 36.1. Biological Agents Cited as Possible Weapons for Use Against Humans, Animals, or Plants

Type of Agent	Name	Pathogenic for			Australian List (Core)[a]	BWC Draft Protocol[b]	CDC List[c]	Select Agent[d]	BSL[e]
		Humans	Animals	Plants					
Viruses	African horse sickness virus		X					USDA	3
	African swine fever virus		X		X			USDA	3
	Akabane virus		X					USDA	3
	Avian influenza virus (highly pathogenic)		X		X			USDA	2/3[f]
	Bluetongue virus		X		X			USDA	2
	Camel pox virus		X					USDA	2
	Chikungunya virus	X			X				3
	Classical swine fever virus (hog cholera virus)		X		X			USDA	3[f]
	Congo-Crimean hemorrhagic fever virus	X			X	X	C	HHS	4
	Dengue fever virus	X			X			HHS/USDA	2
	Eastern equine encephalitis virus	X	X		X	X	B	HHS/USDA	2
	Ebola virus	X			X	X	A	HHS	4
	Foot-and-mouth disease virus		X		X			USDA	3[f]
	Goat pox virus		X		X			USDA	2
	Hantaan virus	X			X				3
	Hendra virus	X	X		X			HHS/USDA	3/4
	Herpes virus (Aujeszky's diasease)		X		X				2/3[f]
	Japanese encephalitis virus	X	X		X			USDA	3
	Junin virus	X			X	X	A	HHS	3/4
	Kyasanur Forest virus	X			X		C	HHS	4
	Lassa fever virus	X			X	X	A	HHS	4
	Louping ill virus		X		X				3
	Lumpy skin disease virus		X		X			USDA	3
	Lymphocytic choriomeningitis virus	X			X				2/3[f]
	Machupo virus	X			X	X	A	HHS	4

(Contined)

TABLE 36.1. *(Contined)*

Type of Agent	Name	Pathogenic for			Australian List (Core)[a]	BWC Draft Protocol[b]	CDC List[c]	Select Agent[d]	BSL[e]
		Humans	Animals	Plants					
	Malignant catarrhal fever virus		X					USDA	3
	Marburg virus	X			X	X	A	HHS	4
	Menangle virus		X					USDA	3
	Monkeypox virus	X	X		X	X		HHS	2
	Murray Valley encephalitis virus	X			X				3
	Newcastle disease virus		X		X			HHS	3
	Nipah virus	X	X		X		C	HHS/USDA	3/4
	Omsk hemorrhagic fever virus	X			X		C	HHS	4
	Oropouche virus	X			X				3
	Peste des petits ruminants virus		X		X			USDA	3
	Procine enterovirus type 9 (swine vesicular disease virus)		X		X			USDA	3
	Potato Andean latent tymovirus			X	X				?
	Potato spindle tuber viroid			X	X				?
	Powassan virus	X			X				3
	Pulmonary & renal syndrome-hemorrhagic fever viruses (Seoul, Dobrava, Puumala, Sin Nombre)	X			X	X	C		3
	Rift valley fever virus	X	X		X	X		HHS/USDA	3
	Rinderpest virus		X		X			USDA	3
	Rocio virus	X			X				3
	St. Louis encephalitis virus	X			X				3
	Sheep pox virus		X		X			USDA	2
	South American hemorrhagic fever (Sabia, Flexal, Guanarito)	X			X		A	HHS	4
	Teschen disease virus		X		X				2
	Tick-borne encephalitis virus	X			X	X	C	HHS	4

	(Russian Spring-Summer encephalitis virus)								
	Variola virus (including Alastrim)	X			X	X	A	HHS	4
	Venezuelan equine encephalitis virus	X	X		X	X	B	HHS/USDA	3
	Vesicular stomatitis virus (exotic)		X		X			USDA	2/3[f]
	Western equine encephalitis virus	X	X		X	X	B		2
	Yellow fever virus	X			X	X	C		3
Bacteria	*Bacillus anthracis*	X	X		X	X	A	HHS/USDA	2/3
	Bartonella quintana	X			X				2
	Botulinum neurotoxin producing species of *Clostridium*	X	X					HHS/USDA	2
	Brucella abortus	X	X		X	X	B	HHS/USDA	2/3
	Brucella melitensis	X	X		X	X	B	HHS/USDA	2/3
	Brucella suis	X	X		X	X	B	HHS/USDA	2/3
	Burkholderia mallei	X	X		X	X	B	HHS/USDA	3
	Burkholderia pseudomallei	X	X		X	X		HHS/USDA	2/3
	Chlamydia psittaci	X	X		X	X			2/3
	Clavibacter michiganensis			X	X				
	Clostridium botulinum	X	X		X				2/3
	Clostridium perfringens, epsilon toxin producing types	X			X				2
	Cowdria ruminantium		X					USDA	
	Coxiella burnetii	X	X		X	X	B	HHS/USDA	2/3
	Enterohemorrhagic *Escherichia coli,* serotype O157 and other verotoxin producing types	X			X		B		2
	Francisella tularensis	X	X		X	X	A	HHS/USDA	2/3
	Liberobacter africanus			X				USDA	
	Liberobacter asiaticus			X				USDA	
	Mycoplasma capricolum (M. mycoides capri)		X		X			USDA	
	Mycoplasma mycoides mycoides		X		X			USDA	
	Ralstonia solanacearum, race 3, biovar 2			X	X			USDA	

(*Continued*)

TABLE 36.1. *(Contined)*

Type of Agent	Name	Pathogenic for Humans	Pathogenic for Animals	Pathogenic for Plants	Australian List (Core)[a]	BWC Draft Protocol[b]	CDC List[c]	Select Agent[d]	BSL[e]
	Rickettsia prowazeki	X			X	X		HHS	2/3
	Rickettsia rickettsii	X			X	X		HHS	2/3
	Salmonella typhi	X			X				2/3
	Shigella dysenteriae	X			X		B		2
	Vibrio cholerae	X			X		B		2
	Xanthomonas albilineans			X	X				1/2
	Xanthomonas campestris pv. *citri*			X	X				1/2
	Xanthomonas oryzae pv. *oryzicola*			X	X			USDA	1/2
	Yersinia pestis	X			X	X	A	HHS	2/3
Toxins	Abrin	X			X			HHS	2/3
	Aflatoxins	X			X				2/3
	Botulinum toxins (neurotoxins)	X	X		X		A	HHS/USDA	2/3
	Clostridium perfringens toxins (i.e, epsilon toxin)	X	X		X		B	HHS/USDA	2/3
	Conotoxins	X			X			HHS	2/3
	Diacetoxyscirpenol toxin	X			X			HHS	2/3
	HT-2 toxin	X			X				2/3
	Microcystin (cyanginosin)	X			X				2/3
	Modeccin toxin	X			X				2/3
	Ricin	X			X		B	HHS	2/3
	Saxitoxin	X			X			HHS	2/3
	Shiga toxin	X			X			HHS/USDA	2/3
	Staphylococcus aureus toxins (i.e., enterotoxins)	X	X		X		B	HHS/USDA	2/3
	T-2 toxin	X	X		X			HHS/USDA	2/3
	Tetrodotoxin	X			X			HHS	2/3
	Verotoxin	X			X				2/3
	Volkensin toxin	X			X				2/3
Prions	Bovine spongiform encephalo-								
pathy agent		X					USDA	2/3	
Fungi	*Coccidioides immitis*	X	X					HHS/USDA	2/3
	Coccidioides posadasii	X						HHS	2/3

	Colletotrichum coffeanum var. *virulans*			X	X			1/2
	Cochliobolus miyabeanus			X	X			1/2
	Microcyclus ulei			X	X			1/2
	Peronosclerospora phillippinesis			X	X		USDA	1/2
	Puccinia graminis			X	X			1/2
	Puccinia striiformis			X	X			1/2
	Pyricularia grisea			X	X			1/2
	Pyricularia oryzae			X	X			1/2
	Sclerophthora rayssiae var. *zeae*			X	X		USDA	1/2
	Synchytrium endobioticum			X	X		USDA	1/2
	Xylella fastidiosa (citrus variegated chlorosis strain)			X	X		USDA	1/2
Protozoa	*Cryptosporidium parvum*	X	X			B		2
	Naeglaeria fowleri	X						2

[a]http://www.australiagroup.net/en/control_list/bio_agents.htm.

[b]Agents for use against humans from: Ad Hoc Group of the States Parties to the Convention on the Prohibition, Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, document BWC/AD HOC GROUP/56-2, at 465-466, which is in Annex A of the Chairman's Composite Text for the BWC Protocol.

[c]Centers for Disease Control and Prevention: Biological and Chemical Terrorism: Strategic Plan for Preparedness and Response. Recommendations of the CDC Strategic Planning Workgroup, Morbidity and Mortality Weekly Report 49, No. RR-4 (2000): 1–14.

[d]USDA (http://www.aphis.usda.gov) and HHS (http://www.cdc.gov/od/sap) Select Agent programs.

[e]Where applicable, data were extracted from: U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical laboratories*, 4th edition, U.S. Govt. Printing Office, Washington, D.C. (1999) and A.K. Vidaver and S. Tolin, In: *Biologic Safety. Principles and Practices* 3rd Ed., D.O. Fleming and D.O. Hunt (editors), ASM Press, Washington, D.C. (2000). Biosafety levels are for work in the laboratory.

[f]BSL-3 Agriculture.

such as *B. anthracis* and variola virus relies on a series of assumptions, which results in a degree of uncertainty in the calculated WP.

36.4 IMPACT OF BIOTECHNOLOGY

Because many agents can be engineered, including some that are currently not threats, the potential for genetic engineering remains a serious concern. Biotechnology has had a tremendous impact on the development of medicines, vaccines, and in the technologies needed to counter the threat of naturally occurring disease. However, biotechnology can also be used for the development of biological weapons (i.e., "dual-use technologies"). Several examples are presented below for illustrative purposes.

36.4.1 Modification of Threat Agents

36.4.1.1 Vaccine evasion Pomerantsev et al. [88] cloned the *Bacillus cereus* cereolysin AB genes, which encode a functional hemolysin, into the fully virulent *B. anthracis* strain H-7. Hamsters immunized with a live anthrax vaccine strain (i.e., *B. anthracis* STI-1 [$pXO1^+$ $pXO2^-$]) were protected when challenged with *B. anthracis* H-7. In contrast, hamsters immunized with *B. anthracis* STI-1 and challenged with the recombinant cereolysin-producing strain of *B. anthracis* were not protected. This "vaccine breakthrough" could be overcome if the cereolysin genes were cloned into the strain used for immunization. This publication brought into question the utility of vaccinating the entire US military population and whether simple genetic modification of *B. anthracis* might circumvent that program.

36.4.1.2 Antibiotic resistance *B. anthracis* [59], *Yersinia pestis* [59], *Francisella tularensis* [37], and *Brucella* species [13] are generally susceptible to most of the antibiotics used to treat these infections. In general, development of antibiotic resistance resulting from gene transfer between organisms occurs in nature at much greater frequencies than in the laboratory. Recently, plasmid-mediated multidrug resistance has been reported in a strain of *Y. pestis* that was isolated in 1995 from a 16-year-old boy in Madagascar [44]. What was particularly alarming was that this strain was resistant to not only all of the antibiotics recommended for therapy (chloramphenicol, streptomycin, and tetracycline) and prophylaxis (sulfonamides and tetracycline) of plague (Fig. 36.2) but also to drugs that may represent alternatives to classic therapy (ampicillin, kanamycin, spectinomycin, and minocycline). The Soviet biological weapons program was purported to have developed recombinant organisms that were resistant to antibiotics as part of their research to develop offensive biological weapons for which there was no current cure [6, 81]. The possibility that they were developing antibiotic-resistant agents is supported, in part, by publications in the open literature dealing with *Brucella abortus* [47], *B. anthracis* [87], *Burkholderia pseudomallei* [1, 2], and *Burkholderia mallei* [1, 110].

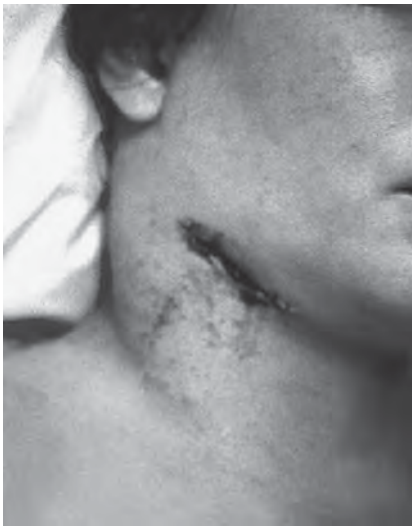

Fig. 36.2. Plague patient whose symptoms include this swollen, ulcerated cervical lymph node. After the incubation period of 2–6 days, symptoms of plague appear including severe malaise, headache, shaking chills, fever, and pain and swelling, or adenopathy, in the affected regional lymph nodes, also known as buboes [provided courtesy of CDC].

36.4.1.3 Bioregulator production The possible use of bioregulators, which are natural substances that control cell and body physiological functions (e.g., bronchoconstriction, vasodilatation, muscle contraction, heart rate, and immune responses) for bioterrorism, has been a concern [109]. Under normal conditions, bioregulators are produced in low concentrations by the host. However, expressing the gene encoding a bioregulator in a suitable bacterial vector can enhance its synthesis. To this end, Borzenkov et al. [15] introduced a genetic construct encoding human β-endorphin into an attenuated strain of *F. tularensis*. The production of the recombinant bioactive peptide produced dose-dependent effects, consistent with endorphin expression, in mice that were infected with the recombinant strain of *F. tularensis*. Borzenkov et al. [16] also constructed recombinant strains of *Yersinia* and *Brucella* capable of producing human endorphin. It is unknown what real effects these organisms might have in humans; however, it has been postulated that they might produce altered pathology following infection that might confound diagnosis and treatment [72].

36.4.2 Modified Low Virulence or Nonpathogenic Organisms

In nature, organisms that are of low pathogenicity but that encode some factors linked with virulence could be converted to more virulent organisms by the acquisition of genes through processes such as lateral gene transfer [80]. A similar process has been used to explain the relatively recent emergence of *Y. pestis* from *Yersinia pseudotuberculosis* [3]. These two organisms are highly related at the genomic level, but cause vastly different diseases [71].

The completion of whole genome sequences of a number of pathogenic microorganisms [41] has increased our knowledge of the genes responsible for virulence. It is possible that someone could use this knowledge to enhance the virulence of another organism. For example, although of natural occurrence, genes encoding anthrax toxin have been found in certain strains of the closely related species, *B. cereus*, which were associated with human illness resembling inhalation anthrax [54].

36.4.2.1 Mousepox virus A recent report from an Australian research group [61] was deemed to have important implications for the genetic engineering of viruses. Jackson et al. [60] were investigating virally vectored immunocontraceptive vaccines based on the orthopox virus, ectromelia virus. This virus is a natural pathogen of laboratory mice that causes a generalized disease termed mousepox. Some inbred mouse strains (e.g., C57BL/6) are genetically resistant due to an effective natural killer (NK) response and the early onset of a strong $CD8^+$ cytotoxic T lymphocyte response, and exhibit subclinical infection with lower levels of viral replication. Jackson et al. [61] were investigating the ability of co-expressed cytokine IL-4 to enhance the antibody-mediated response to other recombinant antigens carried on the virus vector. Instead, these researchers found that the ectromelia virus vector expressing IL-4 altered the host's immune response to this virus resulting in lethal infections in normally genetically resistant mice. More troubling was their observation that this virus also caused lethal infections in mice previously immunized against infection with ectromelia virus. The creation of this "supermousepox" virus led to speculation that similar genetic engineering could be performed on smallpox (Fig. 36.3) leading to a biological weapon that would be effective against an immunized population. The publication of this article also initiated discussions concerning the implication of scientific publications for national security and the responsibilities of journals [9].

36.4.3 Recreation or In Vitro Synthesis of Viral Pathogens

36.4.3.1 Spanish influenza The influenza pandemic of 1918–1919 was uniquely severe, causing an estimated 20–40 million deaths globally [34]. This pandemic happened before the advent of viral culture and very little was known about the virus until recently [82]. The molecular analysis of the virus responsible for the 1918 pandemic has been undertaken using reverse transcription-PCR (RT-PCR) amplification of viral RNA sequences from formalin-fixed and frozen tissue samples in an effort to shed light on both the reasons for its extraordinary virulence and evolutionary origin [11, 89, 90, 100]. Once the entire sequence is known it will become possible to recreate the virus in the laboratory using reverse genetics [52, 82]. Many scientists have stated that such a study should be attempted only if its benefits warrant the risk and if high-level biosafety laboratories are used [111]. Of more immediate concern is whether these same technologies will be used to make H5N1 avian influenza viruses that can be transmitted from human to human.

36.4.3.2 Synthetic genomes Cello et al. [26] synthesized a full-length poliovirus complementary DNA (cDNA) (*ca.* 7500 bp) by assembling oligonucleotides of plus and minus strand polarity. The synthetic poliovirus cDNA was transcribed by RNA polymerase into viral RNA, which translated and replicated in a cytoplasmic extract of uninfected HeLa S3 cells, resulting in the de novo synthesis of infectious poliovirus. Smith et al. [97] were able to assemble up to 130 pieces of synthetic DNA into one long double-helical molecule representing the complete infectious genome of bacteriophage ΦX174 (5386 bp). The final assembly was accomplished in only 14 days. No one is suggesting that poliovirus (at least at this time) or ΦX174 would make good weapons, but these articles raise concerns that

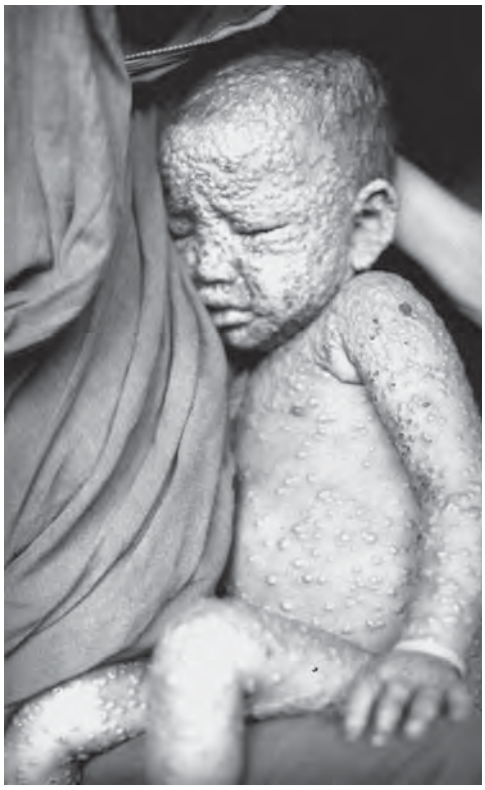

Fig. 36.3. This young Bangladesh child, photographed in 1975, manifested a severe, probably fatal smallpox viral infection with a maculopapular rash that was so dramatic, and the pustules so plentiful that they began to coalesce on his skin, especially on his face. There are four types of variola major smallpox: "ordinary" (the most frequent type, accounting for 90% or more of cases); "modified" (mild and occurring in previously vaccinated persons); "flat"; and "hemorrhagic" (both rare and very severe). Historically, variola major has an overall fatality rate of about 30%; however, flat and hemorrhagic smallpox usually are fatal. Variola minor is a less common presentation of smallpox, and a much less severe disease, with death rates historically of 1% or less [provided courtesy of Stanley O. Foster, M.D., M.P.H., CDC and World Health Organization].

more complicated viruses (e.g., smallpox or Ebola) could be synthesized from scratch based on publicly available sequences, or that viruses could be created that do not exist in the wild [33].

Using another approach, the large genomes of a baculovirus [74], several herpes viruses [14, 35, 76], and vaccinia virus [38] have been cloned as bacterial artificial chromosomes (BACs). These circular miniF BAC plasmids allow viral genomes to be stably maintained at low copy number and manipulated in *Escherichia coli* and then reconstituted as infectious virus by transfection of eukaryotic cells. Using this or a similar approach, one might be able to use *E. coli* to deliver a virus to a susceptible host. Based on the open publication of these advances in biotechnology, some have raised the concern that scientific journals could become cookbooks for terrorists by providing easy recipes for creating scenarios and assembling pathogens that could be unleashed easily on people [62]. An example of this concern is the recently published paper by Wein and Liu [112].

36.4.4 Unintended Consequences of Biotechnology

The following examples will highlight how the results of legitimate research might be used for nefarious purposes.

36.4.4.1 Smallpox virus protein which inhibits complement enzymes Rosengard et al. [93] characterized the orthopox complement regulatory proteins (CRPs) of variola major and vaccinia virus, two closely related viruses, and compared their effectiveness in overcoming human complement activation. The vaccinia virus CRP is secreted by vaccinia-infected cells; it is likely, but not proven, that the variola major CRP is also secreted by variola-infected cells. Only 11 amino acid differences exist between the CRPs from variola and vaccinia; however, the variola CRP was 100-fold more effective than the vaccinia CRP in inactivating both the classical and alternative complement pathways. The authors hypothesized that the variola CRP may create a microenvironment around variola-infected cells to protect them from complement-mediated attack while they serve as a site for viral replication. A concern is that the small differences that exist between variola and vaccinia virus might be bridged by genetic engineering.

36.4.4.2 Encapsulation method Loscertales et al. [73] reported that they had developed a method to generate steady coaxial jets of immiscible liquids with diameters in micrometer/nanometer size. The eventual jet breakup resulted in an aerosol of monodisperse compound droplets with the outer liquid surrounding or encapsulating the inner one. This approach was used to microencapsulate aqueous solutions with diameters varying between 0.15 and 10 microns, depending on the running parameters. Though not intended by the authors, this method could be used for the micro-encapsulation of biological agents for aerosol release.

36.5 SCENARIOS

There are significant differences between biological and chemical terrorism [49]. The important distinctions between these forms of terrorism are presented in Table 36.2. In general, bioterrorist attacks occur as one of two scenarios, that is, covert and overt. It is possible to introduce biological agents into civilian populations by several means including aerosol, contaminated food [68, 98, 112], water [21], or medical products, or by releasing infected arthropod vectors [36]. Because we currently lack the ability to conduct real-time monitoring for the release of a biological agent, an unannounced (i.e., covert) release of a biological agent into a civilian population would likely go unnoticed for some time, with those exposed leaving the area long before the act of terrorism becomes evident. In this respect, it differs from what would happen in a chemical attack (Table 36.2). Due to an incubation period, the first signs that a biological agent has been released may not become apparent until days or weeks later, when individuals become ill and seek medical care. Thus the "responders" to a covert release of a biological agent will likely be the astute clinician, laboratory or public health worker who recognizes the index case or identifies the responsible agent. The "responders" to a covert release affecting animals or plants will likely be a veterinarian, county agent, field agronomist, seed inspector, or elevator operator.

The most likely responders to an announced (i.e., overt) release of a biological agent (or more likely a hoax) are law enforcement or hazmat personnel and firefighters because of their terrorism training. Thus, the initial recognition and response to the deliberate release of a biological agent, whether overt or covert, would occur at the local level.

A comprehensive public health response to the deliberate release of a biological agent (or for that matter, to any outbreak of infectious disease) will involve epidemiologic investigation, medical treatment and prophylaxis for affected persons, and the initiation of disease prevention activities. Analogous activities would occur in a response to a deliberate release of a biological agent affecting animals (epidemiological investigations, treatment, vaccination, culling, or slaughtering) or plants (epidemiological investigations, treatment) [113]. The success of these activities is dependent, to a large extent, upon the rapid and accurate identification of the biological agent.

36.6 RESPONSES TO BIOTERRORISM: LABORATORY

From the inception of efforts to enhance preparedness, there have been differences of opinion on how the laboratory response to bioterrorism should be organized. Some individuals have suggested that a single high-throughput laboratory or perhaps a few regional laboratories would be sufficient to detect and respond to a bioterrorist attack in the United States [70]. However, an effective public health response would have to be rapid. To facilitate the rapid identification

TABLE 36.2. Important Distinctions Between Biological and Chemical Terrorism (Modified from [49])

Criterion	Biological Terrorism	Chemical Terrorism
Speed at which attack results in illness	Delayed – usually days to weeks after attack	Rapid – usually minutes to hours after attack
Distribution of affected patients	Widely spread through city or region; major international epidemic in worst-case scenario	Downwind area near point of release
First responders	Emergency department nurses and physicians, infectious disease physicians, infection control practitioners, epidemiologists, public health practitioners, hospital administrators, and laboratory experts	Paramedics, police, firefighters, emergency rescue, law enforcement
Release site of weapon	Difficult to identify; probably not possible or useful to cordon off area of attack	Quickly discovered; possible and useful to cordon off area of attack
Decontamination of patients and environment	Not necessary in most cases	Critically important in most cases
Medical interventions	Vaccines and/or antibiotics	Chemical antidotes
Patient isolation/quarantine	Crucial if easily communicable disease is involved (e.g., smallpox); advance hospital planning for isolating large numbers of patients is critical	After decontamination there is no need

of threat agents, the Laboratory Response Network (LRN) was created. Operational in August 1999, the LRN was established in recognition that the existing infrastructure of diagnostic testing laboratories competent to deal with biological (or chemical) terrorism in the United States was extremely limited. CDC developed the LRN in concert with the Association of Public Health Laboratories (APHL) and with collaboration from the Federal Bureau of Investigation (FBI) and the US Army Medical Research Institute for Infectious Diseases (USAMRIID). The LRN was conceived to build on the existing interaction of nationwide public health laboratories, which participate in routine disease surveillance activities [45]. In addition, it has a dual function in that it has the ability to detect and respond to agents released by a bioterrorist as well as those that occur naturally, including newly emerging infectious diseases such as SARS [29]. This capacity is particularly important, as it will generally not be known at the time of detection whether the outbreak was intentional or natural.

Initially, the LRN operated as a national network of laboratories designated Level A through Level D. Each of these levels represented progressively stringent levels of safety, containment, and technical proficiency necessary to perform the essential tests to rule out, rule in, as well as perform the referral functions that were required for agent identification and confirmation. Level A laboratories were, for the most part, hospital and other community clinical laboratories. The reasoning behind this decision was that in the aftermath of a covert bioterrorism attack, patients would seek care at many widely dispersed hospitals where these laboratories exist [45]. The primary role that was envisioned for these clinical laboratories was to "raise the level of suspicion" when a critical agent (Table 36.1) was suspected in a clinical specimen [65]. To facilitate this process, protocols and algorithms were developed [7] that could be implemented by the clinical laboratory in its routine day-to-day operation. If a critical agent could not be ruled out, the culture was referred to the nearest LRN Level B or C laboratory. At the beginning of the program, Level B laboratories were primarily state and local public health laboratories with biosafety level (BSL)-2 facilities where BSL-3 practices [27] are observed. Level C laboratories were primary public health laboratories with BSL-3 facilities, or those with certified animal facilities, which are necessary for performing the mouse toxicity assay for botulinum toxin. The Level D laboratories (CDC and USAMRIID) had BSL-4 facilities that could handle agents such as Ebola, for which other laboratories had insufficient safety facilities. The LRN now consists of international members as well as those in the United States.

Recently, the structure of the LRN has changed, in part, because of improvements made as a result of the bioterrorism preparedness funds provided by the US Congress to strengthen the public health infrastructure. The classification of Level A –D laboratories have now been replaced by sentinel (formerly Level A), reference (formerly Levels B and C), and national (formerly Level D) laboratories [78]. There are

currently 148 LRN reference laboratories that are located in the United States, Canada, Australia, United Kingdom, Mexico, Germany, and South Korea.

To ensure consistent results, the reference laboratories use standard protocols and reagents for the identification and confirmation of threat agents. These protocols are available on a secure web site, which also contains the information for ordering the necessary reagents and control strains. Due to the lack of commercially available reagents, the LRN currently relies on those manufactured at CDC or obtained from one of its partners.

Prior to the availability of funds for bioterrorism preparedness, many public health laboratories in the United States were technologically lagging behind those in the private sector. The LRN has facilitated the introduction of state-of-the-art rapid technologies, for example, real-time polymerase chain reaction assays [53] and time-resolved fluorescence immunoassays [85] into the public health sector. These new assays have markedly reduced the amount of time it takes to make an identification of a critical agent. For example, culture may require 24 –72 h for the growth and identification of the agent; using direct fluorescent antibody staining, some agents can be identified in about 4 h. Today, using some of the technologies available in LRN laboratories, an answer can be obtained in less than 2 h. The threat of bioterrorism has also stimulated the development of rapid technologies and assays for use in the field [42] as well as the clinical laboratory [43, 57]. A number of new and promising technologies including genetically engineered B lymphocytes [91], micromechanical sensors [64, 84], fluorescence-based sensors [84], and planar waveguide array biosensors [95] have also been developed for the rapid detection of threat agents and toxins. However, additional work is needed before these technologies are used in laboratories or in the field.

36.7 RESPONSES TO BIOTERRORISM: EPIDEMIOLOGY AND SURVEILLANCE

The deliberate nature of the dissemination of a biological agent will often be obvious, as in the case of multiple mailed letters containing highly refined anthrax spores [63] (see Fig. 36.1). However, in some instances, some forms of bioterrorism may be more covert and very difficult to distinguish from a naturally occurring outbreak. For example, the deliberate contamination of salad bars in The Dalles, Oregon in 1984 by a religious cult in an effort to test their ability to incapacitate the local population prior to an election resulted in multiple point-source outbreaks and sickened more than 750 persons [104]. The outbreak of salmonellosis was specifically excluded as bioterrorism during the initial investigation, and only recognized as such following a tip from an informant [23]. Given the natural ability of infectious diseases to emerge, the Oregon outbreak serves to highlight the difficulties in determining a characteristic signature for an infectious disease outbreak resulting from deliberate introduction of a biological agent. The investigation of the outbreak revealed a very unusual pattern of infections with a rare strain of *Salmonella* Typhimurium. Although the possibility of intentional contamination was considered early in the investigation, it was specifically excluded for the following reasons: (i) such an event had never been reported previously; (ii) no one claimed responsibility; (iii) no disgruntled employee was identified; (iv) no motive was immediately apparent; (v) the epidemic curve suggested multiple exposures, which was presumed to be unlikely behavior for a saboteur; (vi) law enforcement officials failed to establish a recognizable pattern of unusual behavior; (vii) a few employees had onset of infection before the patrons suggesting a possible source of infection; (viii) the outbreak was biologically plausible – even if highly unlikely; and (ix) it is not unusual not to be able to find a source in even highly investigated outbreaks. Although one of the initial reasons to exclude terrorism (i.e., no prior incidents) is no longer applicable, determining if an unusual outbreak is biologically plausible will remain a challenge. For example, the index case of inhalation anthrax (Fig. 36.4) identified in Florida in 2001 was initially thought to be natural [63]. It is also clear from these two documented incidents of bioterrorism – the 1984 Oregon salmonella outbreak [104] and the 2001 anthrax attack [63] – that a terrorist will not necessarily announce his/her intentions to take credit for such an attack.

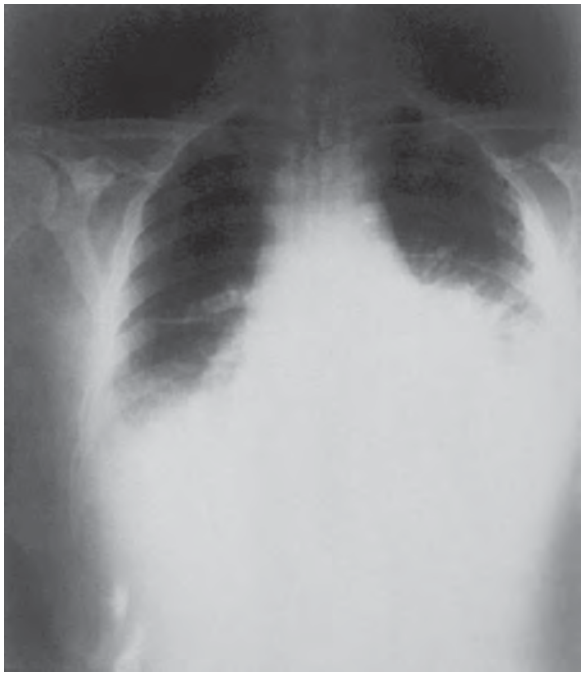

Fig. 36.4. Chest radiograph showing widened mediastinum due to inhalation anthrax. This radiograph taken 22 h before death; AP Chest X-ray. Many of the victims of the anthrax 2001 attack who developed inhalational anthrax had similar appearing chest radiographs [provided courtesy of CDC and P.S. Brachman, M.D.].

Similarly, divining the motives behind an attack should be abandoned as a public health tool to assess whether an outbreak is natural or deliberate in nature. Fortunately, there are a number of epidemiologic clues that alone or in combination may suggest that an outbreak is deliberate. It is essential to make this determination, not only from the law enforcement standpoint, but to protect the public (or animal/plant) health. There is a very short "window of opportunity" in which to implement postexposure prophylaxis (or to minimize the economic impact of an attack on agriculture) for many of the agents likely to be used for bioterrorism [66, 113].

A set of epidemiologic clues based on distinctive epidemiology and laboratory criteria of varying specificity (Table 36.3) have been proposed to evaluate whether an outbreak may be of deliberate origin [105]. The clues focus on aberrations in the typical characterization of an outbreak by person, place, and time, in addition to consideration of the biological agent. Some of the clues, such as a community-acquired case of smallpox (see Fig. 36.3), are quite specific for bioterrorism, whereas others, such as a similar genetic type of an organism, may simply denote a natural outbreak. A combination of clues, especially those that suggest suspicious point-source outbreaks, will increase the probability that an outbreak is likely due to bioterrorism. While these clues were developed with humans in mind, many are applicable to bioterrorist attacks on agriculture.

Surveillance to identify increases in disease incidence is the first step and the cornerstone of bioterrorism epidemiology [17]. The majority of the clues described in Table 36.3 simply suggest an unusual cluster of cases. Treadwell et al. [105] have reorganized these clues by specificity to trigger increasingly broader investigations by state and federal public health officials and to alert law enforcement authorities. However, even the most specific of clues (e.g., uncommon agent or disease) may signal a new natural outbreak. In one

TABLE 36.3. Epidemiologic Clues that may Signal a Biologic Terrorist Attack (Modified from [105])

Single case of disease caused by an uncommon agent (e.g., smallpox, viral hemorrhagic fever, inhalation or cutaneous anthrax, glanders, foot and mouth disease) without adequate epidemiologic explanation
Unusual, atypical, genetically engineered, or antiquated strain of an agent (or antibiotic resistance pattern)
Higher morbidity and mortality in association with a common disease or syndrome or failure of such patients (or animals or plants) to respond to usual therapy (or treatments)
Unusual disease presentation (e.g., inhalation anthrax, pneumonic plague)
Disease with an unusual geographic or seasonal distribution (e.g., plague in a non-endemic area, influenza occurring in the northern hemisphere in the summer)
Stable endemic disease with an unexplained increase in incidence (e.g., tularemia, plague)
Atypical disease transmission through aerosols, food, or water, in a mode suggesting sabotage (i.e., no other possible physical explanation)
No illness in persons who are not exposed to common ventilation systems (have separate closed ventilation systems) when illness is seen in persons in close proximity who have a common ventilation system
Several unusual or unexplained diseases coexisting in the same patient (or animal, or plant) without any other explanation
Unusual illness that affects a large, disparate population (e.g., respiratory disease in a large heterogeneous population may suggest exposure to an inhaled biological agent)
Illness that is unusual (or atypical) for a given population or age group (e.g., outbreak of measles-like rash in adults)
Unusual pattern of death or illness among animals (which may be unexplained or attributed to an agent of bioterrorism) that precedes or accompanies illness or death in humans
Unusual pattern of death or illness in humans that precedes or accompanies illness or death in animals (which may be unexplained or attributed to an agent of bioterrorism)
Ill persons who seek treatment at about the same time (point source with compressed epidemic curve)
Similar genetic type among agents isolated from temporally or spatially distinct sources
Simultaneous clusters of similar illness in noncontiguous areas – domestic or foreign
Large numbers of cases of unexplained diseases or deaths

example, the recent community outbreak of individuals with smallpox-like lesions in the mid western Unite States may have indicated the deliberate release of smallpox virus. However, a thoroughly integrated epidemiologic and laboratory investigation identified the disease as monkeypox, an exotic disease in the United States, which in itself should suggest bioterrorism [30]. Affected individuals were infected by prairie dogs purchased as pets, which had acquired their infections while co-housed with infected Giant Gambian rats that had recently been imported from Ghana and not from deliberate dissemination. In another example, the diagnosis of bubonic plague in two visitors to New York City [31] may have indicated an act of bioterrorism, especially because plague had not occurred in this city in more than 100 years. However, a thorough epidemiologic investigation determined that the exposure occurred in Santa Fe County, New Mexico, where plague is endemic and, as a result, an act of bioterrorism was ruled out.

There have been other approaches used to differentiate a natural epidemic from one due to bioterrorism. For example, Grunow and Finke [48] developed a weighted list of 11 criteria (e.g., biological risk, biological threat, agent, geographic distribution of agent, concentration of agent in the environment, etc.), with points allocated to represent their importance, which enabled them to deduce in a semi quantitative manner the possibility of an intentional outbreak. This approach needs to be evaluated further.

36.8 MOLECULAR EPIDEMIOLOGY AND MICROBIAL FORENSICS

Extremely sensitive and specific molecular techniques have recently been developed to facilitate epidemiologic studies [92]. Our ability to use these genotypic methods to detect and characterize the genetic variability of infectious agents (viruses, bacteria, fungi, protozoa) is the foundation for the majority of molecular epidemiological studies. The application of appropriate molecular techniques has been an aid in the surveillance of infectious agents and in determining sources of infection. In some cases, molecular data can effectively rule out an outbreak and thus avoid the need for an extensive epidemiological investigation. In other cases, these data may reveal the presence of outbreaks caused by more than one strain. Molecular techniques can be used to study health and disease determinants in animal (including human) and plant populations. It requires choosing a molecular method(s) that is capable of discriminating genetic variants at different hierarchical levels coupled with the selection of a region of the genome that is appropriate to the questions being asked [103].

The use of molecular methods will play an important role in the investigation of outbreaks resulting from bioterrorism [25, 67]. Unfortunately, the discriminatory power of molecular typing procedures is not well defined. For example, consider the anthrax letters attack where a multilocus variable number tandem repeat (VNTR) analysis was used for *B. anthracis* strain identification [67]. The data were qualitatively interpreted as indicative of the Ames strain and focused the criminal investigation toward a laboratory source; however, no further attribution was possible. With the exception of whole genome sequencing, molecular methods analyze only a small portion of the organisms' genetic complement. For this reason, isolates that give identical results are classified as "indistinguishable," not "identical". In the context of bioterrorism, only whole genome sequencing will provide the unequivocal data needed for attribution [19].

Microbial forensics is a newly emerging field of microbiology, which will aid in the investigation of bioterrorism [18]. Previous experience suggests that nucleic acid-based methods, while extremely important, are unlikely to pinpoint a unique source. Thus, chemical and physical analyses of evidence almost certainly will be needed to obtain additional information. As part of the effort to deter biological terrorism and strengthen the law enforcement response to such an act, the United States recently established a microbial forensic laboratory known as the National Bioforensics Analysis Center (NBFAC), which is part of the Department of Homeland Security (DHS) and operates in partnership with the FBI [19]. The NBFAC provides a central facility to conduct analysis of evidentiary material. However, a number of significant gaps in both science and operations will need to be addressed to further the development of this new field [20]. To address the need for information, a large database has been developed relating microorganism names, taxonomic classifications, diseases, specific detection and treatment protocols, and relevant literature. This Microbial Rosetta Stone Database [41] (http://www.microbialrosettastone.com) facilitates linkage to public genome data bases and will provide valuable information for future investigations.

36.9 BASIC AND APPLIED RESEARCH

The bioterrorism threat has created a conundrum in the United States. On the one hand is the need for legitimate research for biodefense purposes, while on the other is the need to protect the public by restricting illegitimate access to dangerous biological agents. The National Institute of Allergy and Infectious Diseases (NIAID) is the principal institute among the National Institutes of Health, supporting research on infectious agents that represent threats as agents of bioterrorism. Since the anthrax attacks of 2001, NIAID has markedly expanded, intensified, and accelerated its ongoing research programs in biodefense (see also Biron et al. [12]). From a scientific and medical perspective, NIAID has considered bioterrorism to be a variant of the general problem of emerging infectious diseases, the only difference being that increased virulence or spread into a susceptible population is a deliberate act rather than a consequence of natural evolution. The

scientific needs and areas of NIAID research emphasis are currently in the areas of basic biology of the microbe, host response, vaccines, therapeutics, and diagnostics [108]. The agents of concern were not limited to those in CDC Category A–C (Table 36.1), but included a number of emerging and reemerging pathogens, such as West Nile Virus, influenza viruses, and drug-resistant *Staphylococcus aureus* that are recognized as having many characteristics that make them potential agents of bioterrorism. In the past, a lack of adequate BSL-3 and BSL-4 laboratories limited research on many of these agents (Table 36.1). Thus, the construction of additional facilities became a priority. NIAID has also funded a number of Regional Centers of Excellence for Biodefense and Emerging Infectious Disease Research (RCEs). The RCEs form a national network of academic centers that conduct research to counter threats from agents of bioterrorism and emerging infectious diseases. Each consortium was established to support investigator-directed research; train researchers, and other personnel for biodefense research activities; provide resources used by other researchers within the region and network; conduct research focused on development and testing of vaccines, therapeutics, and diagnostics; provide core facilities to approved investigators from academia, government, biotech companies, and the pharmaceutical industry; and provide facilities and scientific support to first responders in the event of a national biodefense emergency.

36.10 LIMITING ACCESS TO DANGEROUS PATHOGENS

Pathogenic microorganisms are widely available either from the natural environment in areas where diseases such as anthrax, plague, or tularemia are endemic, or from many of the 1500 culture collections scattered across the globe that provide cultures to commercial entities and biomedical researchers. The situation is further complicated by the fact that some dangerous pathogens and toxins are not only studied by biomedical researchers but have also become commercial products. For example, pharmaceutical companies currently produce large quantities of botulinum toxin (Botox) for medical and cosmetic purposes.

In 1995, Larry Wayne Harris illegitimately obtained lyophilized cultures of *Y. pestis* from the American Type Culture Collection [99]. Partially in response to concerns that dangerous pathogens could be easily accessed for illegitimate purposes, congress passed the Antiterrorism and Effective Death Penalty Act of 1996 (Public Law 104-132), which contained a provision (i.e., Section 511) that was written to limit access to biological agents that could be used by domestic terrorists, or to make a weapon of mass destruction, without inhibiting legitimate research in this area. Section 511 of this law directed the Secretary HHS to (i) maintain a list of biological agents that have the potential to pose a severe threat to public health and safety (i.e., select agents); (ii) establish procedures for the transfer of select agents; (iii) provide safeguards to prevent access to select agents for criminal purposes; (iv) establish procedures to protect public safety in the event of a transfer of a select agent in violation of safety procedures; and (v) ensure availability of agents for legitimate purposes. The implementing regulation, which was termed "The Select Agent Rule", tracked the interstate and intrastate transfer of select agents between registered facilities.

In the aftermath of the anthrax attacks of 2001, the US Congress passed two additional laws dealing with the use or possession of dangerous pathogens. The first was the "Uniting and Strengthening America by Providing Appropriate Tools Required to Intercept and Obstruct Terrorism Act of 2001" (Public Law 107-56), also known as the "USA Patriot Act". The "USA Patriot Act" was a new criminal law, which defined a category termed "restricted persons". Section 175b stated that no "restricted person" shall ship, possess, or receive a select agent (Table 36.1). The second was the "Public Health Security and Bioterrorism Preparedness and Response Act of 2002" (Public Law 107-188), which provided new provisions on the possession, use, and transfer of select agents. The purpose of this Act was to improve the capacity of the United States to prevent, prepare for, and respond to bioterrorism and other public health emergencies.

Section 202(a) of Public Law 107-188 required all persons in possession of biological agents or toxins deemed a threat to public health to notify the Secretary, HHS. Section 213(b) of the Law required all persons in possession of biological agents or toxins deemed a threat to animal or plant health or products to notify the Secretary, USDA. The new regulations, which were promulgated, provide for regulatory oversight of select agents and toxins (expanded to include animal and plant pathogens), and a process for limiting access to persons who have a legitimate need to possess, use, or store these agents. Through a mandatory registration process and a security risk assessment performed by the FBI, national databases have been established of where select agents and toxins are used as well as of individuals authorized to have access to them. The regulations also establish and enforce safety and security procedures for select agents and toxins, including measures to ensure the proper training and appropriate skills to handle them. Ultimately, the goal of these regulations is to ensure appropriate availability of biological agents and toxins for research, education, and other legitimate purposes within the framework of a monitored and secure environment.

Unfortunately, the requirements for registration for possession and the security risk assessment caused some to predict [102] that the new regulation would have a negative impact on biodefense research in the United States; a few investigators destroyed or transferred their select agents rather than register under the regulation. The recent conviction of a US scientist in federal court for illegally transporting cultures of *Y. pestis* and for fraud has demonstrated that the US government will enforce these regulations [77]. Nevertheless, the availability of new grant funds has enticed many new investigators into biodefense research.

The strengthened select agent regulation is an important step in reducing the threat of bioterrorism in the United States. However, it does not address the international dimensions of the problem. Worldwide, thousands of research laboratories and culture collections store dangerous pathogens under insecure conditions [106]. In order to address this issue, the United Nations Security Council adopted Resolution 1540 requiring all member states to implement national legislation to prevent the proliferation of weapons of mass destruction (WMD), including biological weapons. National regulations to ensure the physical protection, control, and accounting of dangerous pathogens and toxins clearly fall under Resolution 1540 [106]. Guidelines or "best practices" for the implementation of Resolution 1540 remain to be developed.

36.11 SUMMARY

The threat of bioterrorism is real. Dealing with this threat presents professional, scientific, legal, and ethical challenges. Steps are being taken to reduce access to dangerous pathogens as well as develop defensive countermeasures. However, advances in biotechnology have expanded both the nature and magnitude of the threat. Considering the accessibility of biotechnology through the open literature, Internet, and commercial entities, the key element in averting bioterrorism and biowarfare may not be access to the agent but intent, whether on the part of individuals, groups, or national governments [75]. An effort to address the element of intent also needs to be intensified if we are to prevent exploiting biotechnology for hostile purposes.

GLOSSARY

Bacteriophage: A virus that infects bacteria.

B-lymphocyte: An immune system white blood cell that carries out the humoral immune response. B-cells are produced in the bone marrow and mature into plasma cells that produce antibodies. B-cells are influenced by various cytokines (chemical messengers) as well as interactions with T-cells and other immune system components.

CD8$^+$ cytotoxic T lymphocyte: A large differentiated T cell that attacks and lyses target cells bearing specific antigens.

Cytokine IL-4: A protein (chemical messenger) that stimulates the immune system to develop mast cells, resting T cells, and activated B cells.

Complementary DNA (cDNA): DNA that is synthesized from a messenger RNA template; the single-stranded form is often used as a probe in physical mapping.

Polymerase chain reaction (PCR): A method of amplifying or copying DNA fragments that is faster than cloning. The fragments are combined with DNA polymerase, nucleotides, and other components to form a mixture in which the DNA is cyclically amplified.

pXO1, pXO2: Two plasmids (extrachromosomal DNA) that are found in virulent strains of *Bacillus anthracis.* The plasmids possess the genes encoding virulence factors such as the toxins and capsule.

Reverse transciption PCR: A method of amplifying RNA by first synthesizing a cDNA copy using the enzyme reverse transcriptase.

SARS: Severe acute respiratory syndrome (SARS) is an atypical form of pneumonia. It first appeared in November 2002 in Guangdong Province, China. SARS is caused by a coronavirus; around 10% of infected people die from it.

T lymphocyte: T cells receive their name from the "t" in thymus, a gland in the chest that shrinks and disappears as people grown into adulthood. The T lymphocytes are derived from the thymus in fetal life, childhood, and young adulthood before it atrophies. These cells are critical to a variety of immune functions. Uncontrolled proliferation after the malignant transformation of this type of cell gives rise to T cell leukemia or lymphoma.

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CHAPTER 37

Needs for an Integrative Approach of Epidemics: The Example of Cholera

R. Piarroux and D. Bompangue

Santé et Environnement Rural, Université de Franche-Comté, EA 2276, Besançon, France

Every year Cholera made a visit to the Holy City of Mecca with her companions Death and Fear. One year however, Fear came to the city before Death and Cholera. The old gatekeeper, who did not know Fear, let her enter. When Cholera and Death arrived at the gate, the gatekeeper shouted, "Cholera, how many victims will you take this time?" "Not more than 500 I'm sure this time," Cholera said. "Death, how many will you take?" the gatekeeper cried. "As always, I will take only what Cholera gives me." The gatekeeper let them enter. Weeks later, Death and Cholera returned and called to the gatekeeper, "Open the gates." "Cholera, how many victims did you take?" the gatekeeper asked. "Only 499," Cholera answered. "And Death, how many did you take?" asked the gatekeeper "I took more than a thousand." "You promised you'd only take what Cholera gave you!" the gatekeeper cried. "Yes," Death answered sadly, "Most of those who died were taken by Fear who entered your gate alone before us. Now you know that Fear does more harm and causes more deaths than Cholera!" [19].

37.1 INTRODUCTION

According to official reports, 100,000–400,000 new cases have been recorded each year during the past two decades [4,5,50]. Most of these cases have been reported during outbreaks occurring in Africa [47]. These data are all the more alarming because cholera is notoriously under-declared, either due to failure to diagnose it or because governments are afraid of the deleterious consequences, which outbreak reports will have on their economy and tourism. Some authors estimate that worldwide, cholera was responsible for more than 6 million cases (recorded and nonrecorded) during the 1990s alone [13]. Due to its obvious links with poverty, malnutrition, poor access to heath facilities, and armed conflict, cholera is one of the main problems that concern medical nongovernmental humanitarian organizations (NGO), and particularly those NGOs intervening in crises and emergencies. However, this is only one of the many aspects of cholera. Cholera is also an infectious disease caused by environmental bacteria, which live in estuarine and brackish waters. The relationship between *Vibrio cholerae*, the causative agent of the disease, and environment has been studied from the molecular level (How have some *V. cholerae* strains acquired the capacity to infect humans and provoke epidemics?) to a planetary level (Do global climatic changes affect the patterns of cholera epidemics?). And last but not least, as shown by the short tale at the beginning of this chapter, the word cholera is often associated in people's minds with the notions of death and fear. This disease is actually one of the rare diseases that has played an influential role in human history, especially during the nineteenth century, affecting rural areas as well as the largest industrialized cities of Europe and North America. At that time, cholera was responsible for panic and irrational behavior, revealing society's most deeply buried fears. With this in mind, cholera in the nineteenth century is comparable to the plague in the Middle Ages. Even now, in 2007, the word "cholera" is still capable of inducing fear in people all over the world, including reporters and NGO volunteers, whose subsequent testimonies cause inappropriate reaction in the general public and politicians. People's beliefs and fears about cholera, their

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behavior during an epidemic, as well as the attitude of medical staff and other actors involved in the fight against the disease, play major roles in the dynamics of epidemics. On a larger scale, public opinion about this disease appears to be one of the main determinants in the willingness of political actors to take action against cholera and to financially support public health programs targeting this disease. To take a closer look at these different aspects of cholera, we will present the causative agent and its natural environment, followed by a description of the disease first from an individual and medical point of view and then from a collective and historical one. We will thereby attempt to account for the impressive fact that this disease still exists, in 2007, despite the considerable medical and scientific progress that has been made over the last two centuries.

37.2 *VIBRIO CHOLERAE* AND ITS NATURAL ENVIRONMENT

37.2.1 *Vibrio Cholerae*

Cholera is caused by bacteria of the *V. cholerae* species. They are gram-negative, curved, and each presents with a single polar flagellum, which provides rapid mobility, a characteristic of the *Vibrio* genus (Fig. 37.1). Cholera is a waterborne disease, and *V. cholerae* has been identified as free-living bacterial flora in brackish water in estuarine areas where it can colonize the surface of algae and plankton [15, 49]. These bacteria existed long before humans; most *V. cholerae* strains are still part of the natural flora of the aquatic environment. Among them, some aquatic clones acquired new genetic information and were then able to colonize the human [55]. Epidemics of cholera are caused by *V. cholerae*, which belong to the O1 or O139 serogroups. In the O1 serogroup, two biotypes, called "classical" and "El Tor" have been identified. They can be differentiated using biological techniques such as the study of biochemical characters or of susceptibility to bacteriophages (viruses infecting bacteria), and resistance to antibiotics such as polymixin B. However, techniques based upon DNA identification are today more generally used in differentiating *V. cholerae* isolates. Besides O1 and O139, there are many other strains, which belong to the species *V. cholerae*, but they do not cause cholera. They are called non-O1 non-O139 strains and can sometimes be isolated in humans who are suffering from benign diarrhea or other pathogenic conditions such as wound infections, or who are immunocompromised and have sepsis [17,21,23,24,31,37,41,43].

Fig. 37.1. *Vibrio cholerae* J. Dowdalls/photo researchers /bsip.

Identification of the species, the serogroup, and the biotype is enough to diagnose the disease. However, from an epidemiological point of view, other tools are required to track cholera clones during an outbreak or to find an environmental origin for an epidemic clone. Basically, each O1 biotype can be subdivided into serotypes, differing in their ability to synthesize A, B, and C antigens. Serotype Ogawa strains synthesize A and B antigens and a low amount of C antigen; serotype Inaba strains only produce A and C antigens, and Hikojima strains synthesize all three antigens. However, this last serotype is rarely encountered. Many other techniques have been successfully used to differentiate cholera isolates, including bacteriophage typing, amplified fragment length polymorphism, multilocus sequence typing, repetetive extragenic palindrome PCR, ribotyping, and whole genome [22,32,33,39].

37.2.2 Biotope of *Vibrio Cholerae*

In nature, *V. cholerae* usually lives in brackish water (salty and alcaline), containing plankton and organic matter. These conditions are encountered in coastal areas, where fresh water from rivers mixes with marine salt water. *V. cholerae* has been found to colonize the surface of algae and copepods, and is capable of existing in such environments during prolonged periods [11, 25]. In their environmental reservoir populations of *V. cholerae* change depending upon certain parameters. In Bangladesh, it has been shown that the frequency of cholera cases increased during the wet season, a warm period during which plankton and algae proliferate [28, 52]. Inversely, if deleterious environmental conditions occur, such as nutriment depletion, lower temperatures, or excess of salt, *V. cholerae* can change into a viable but unculturable form [16, 28]. Estuarine and coastal areas are dynamic ecosystems, in which populations of *V. cholerae* are in equilibrium with other organisms composing plankton. This dynamic aquatic ecology may be affected by human activities through organic and industrial pollution, which, for some authors, can affect the bacteria's virulence, even before it is ingested [13]. The survival of *V. cholerae* on plankton has sometimes led to small outbreaks occurring after consumption of oysters collected from polluted coastal areas [45]. Human and environmental interaction in coastal areas is therefore an important component of the explication of the link between coastal environments, organisms of which are ingested, and cholera incidence [13]. Due to the links existing between *V. cholerae* populations,

physicochemical and biological characteristics of their natural biotope, and their capacity to infect humans, it is likely that human activity and its effect on global climate change may have some impact on cholera epidemiology. Several studies have already shown the relationship between the incidence of cholera in the Bay of Bengal, the temperature of water, and seasonal abundance of plankton in estuarine waters [14, 42]. The Southern Oscillation, a large-scale atmospheric "seesaw" centered over the equatorial of the Pacific Ocean, and the consequences of its activity, called El Nino, a term used to describe an anomaly in the flow of ocean water along the west coast of South America, have also been considered as a key factor in explaining the spread of cholera epidemics during certain years, in Asia, Africa, and South America. The change of atmospheric pressure due to Southern Oscillation activity influences the climate even in regions quite distant from it: Drought in South-East Asia and parts of Africa contrasting with heavy rainfall and flooding in other parts of Africa and South America. Cholera outbreaks can occur in either floods or drought [7,14]. It has been suspected that Southern Oscillation and El Nino played a role in the recrudescence of cholera in East Africa in 1997 and 1998 as well as in South America in 1991 and 1998. The effects of global climatic change provoked by human activity on the range and frequency of Southern Oscillation activity are still questionable, but it has been hypothesized that, in the future, these phenomena will be both more frequent and more intense. However, this does not mean that the risk of cholera will increase in the future, because the spread of cholera epidemics are due to a number of factors, including socioeconomical factors that will also evolve with the time.

37.2.3 VPIΦ, CTXΦ Bacteriophages and Pathogenic Strains of *V. cholerae*

The ability of *V. cholerae* strains to induce cholera in humans depends on the presence, in their genome, of clusters of virulence genes, including the CTXΦprophage [56] and the toxin-coregulated pilus (TCP) pathogenicity island [26]. The genome containing the CTXΦprophage is transmitted to the bacterium *via* a biological event called lysogeny. Lysogeny is a phenomenon in which a bacteriophage infects the bacterium, but rather than killing it, its genome incorporates itself into the bacterial genome, and a new organism is produced which is a recombination of the two genomes. The TCP island contains genes coding for proteins that are involved in the biosynthesis of TCP, a pilus required for colonization of the small intestine [35]. Although the origin of this pathogenicity island is still questionable for some authors [26], Karaolis et al. [36], in a recent paper, gave some substantial arguments showing that the TCP island is probably the genome of a filamentous phage called VPIΦ . Toxigenic *V. cholerae* seems to be the result of successive attacks of some environmental *V. cholerae* by phages, which incorporated their genome, thus producing a bacteria-phage chimera [27]. Toxigenic *V. cholerae* therefore present with a set of virulence characters, including the ability to fix themselves on intestinal mucosa, and a toxin, called cholera toxin, which is responsible for the clinical features of the human disease. This toxin has a molecular weight of 84,000 and is composed of five B subunits and one A subunit [34]. B subunits are involved in the fixation of the toxin on GM1 ganglioside receptors located on the mucosa of the human small intestine. A subunit is responsible for the toxic effect. It is transported into the cell where it activates adenylate cyclase, leading to an increase in cyclic adenosine mono phosphate (cAMP). Chloride secretion is increased and neutral sodium chloride absorption is inhibited, which in turn leads to a massive outpouring of fluids into the small intestine exceeding the absorptive capacity of the bowel, resulting in watery diarrhea [51]. Briefly, cholera is more a toxin-provoked disease than an infectious disease, resulting in watery diarrhea and transmitted by an environmental bacterium, *V. cholerae*, which was previously infected by two phages. One of them is CTXΦ and encodes for the toxin, and the other is VPIΦ, or a similar one, and encodes for other pathogenic factors.

37.3 CHOLERA

37.3.1 Clinical Manifestations

Clinical manifestations of cholera, which are due to the proteic enterotoxin described above, can take a variety of different forms, from the asymptomatic carrier to the "full-blown" syndrome with acute hypotension and almost immediate death. The more vibrios there are, the more toxin is secreted, and thus the more severe are the symptoms. In the classical form, cholera begins 12 h to 5 days after the ingestion of *V. cholerae*, with watery diarrhea, beginning abruptly and rapidly increasing. Stool is singularly afaecal, and quickly takes on the typical "rice-water" appearance: a grey and turgid liquid, with some mucus flecks. This diarrhea can be accompanied by vomiting, muscle cramps, and abdominal pain, but no fever. The other signs of cholera are a result of dehydration and vary with its degree of severity: thirst (loss of 3–5% of body weight), postural hypotension, tachycardia, decrease in skin turgor (5–8%), hypotension and weak or absent pulse, ocular hypotension, wrinkled skin (sunken fontanelles in infants), somnolence and coma (more than 10%). Dehydration is accompanied by anuria, which can only be corrected if the patient is rehydrated. Many patients present with respiratory acidosis with Kussmaul's gasping breathing. A patient with the most acute syndrome dies in a few hours from hypovolemic shock, sometimes even before having diarrhea. Several complications can occur, especially when patients are subjected to inadequate treatment, including acute renal failure, hypoglycaemia, and hypokalaemia [51]. In pregnant women, shock and insufficient perfusion of the placenta may lead to miscarriage or premature delivery [51].

It is important to remember that in epidemic conditions, the sudden outbreak of a disease which can take hold of individuals who are in perfectly good health and, in only a few hours, radically transform them physically, is enough to frighten anyone. Acute dehydration changes the victims into

dried up caricatures of their former selves. Respiratory difficulties and approaching death can be accompanied by cyanosis, which turns the skin blue, and neurological disorders can make the person appear to be possessed. And what if the epidemic hits in the middle of Mardi Gras or Carnival? In a letter to a friend, the German poet Heinrich Heine described an outbreak of cholera in Paris at a masked ball. He wrote, "*Suddenly the most gaily-clad of the harlequins collapsed, his limbs cold and he removed his mask, revealing a face that had turned violet-blue. The laugher died, the dancing ceased, and in just a few minutes the carriages of the guests rushed headlong from the ball to the hospital to die there. To prevent the patients from panicking, the dead were thrown unceremoniously into freshly-dug graves, still wearing their harlequin costumes*."

Cholera, however, does not always present with such a severe form, and during an epidemic, a high percentage of persons infected with the *V. cholerae*, either do not appear ill at all or present with common diarrhea, which the patient does not identify as cholera. This is due, on the one hand, to individual variations in susceptibility to cholera (blood group, gastric acidity, previous immunization, and concomitant infection [53]), and on the other hand, to the quantity of bacteria ingested. While the large percentage of asymptomatic and pauci-symptomatic patients is good from a medical point of view, it is anything but from an epidemiological point of view, because of the repercussions on the dissemination of the germ. Asymptomatic carriers invalidate any attempt at detection for carriers and cripple any attempts to stop transmission. And lastly, asymptomatic carriers allow the bacterium and the CTXΦphage to travel with their new human host without killing or even immobilizing him/her, thereby increasing the chances of colonizing new biotopes: something they could not have done without human help. Thus, some authors have presented the hypothesis that *V. cholerae* O1 colonization of new coastal biotopes in Africa and South America could become a reality, given the epidemic outbreaks that occurred at the end of the twentieth century [39].

37.3.2 Guidelines for Collective Management of a Cholera Epidemic

Here, we will deal only with the general guidelines for the collective management of a cholera epidemic. For more details, we refer the reader to documents put out by WHO, Centers for Diseases Control and the main medical NGOs [3,6,10]. The first priority is to set up centers for the treatment of cholera (CTC). In refugee camps, CTCs are generally built from scratch, but when epidemics occur in the open areas, it is often easier and cheaper to set them up in what already exists, such as designate one building of a hospital complex as the cholera building. A CTC has a dual purpose: Reduce the effects of the disease by establishing early appropriate treatment (mainly rehydration of the sick), and limiting the spread of the germ by isolating the sick. The CTC must be physically isolated from outside by an entry way in which hands and shoes are disinfected. To reduce the comings and goings, toilet facilities and an area for food preparation must be built inside the CTC. The patients should be placed on beds with perforated mattresses, one bucket under the bed and another at the head of the bed. In this way, patients are spared from getting up. Usually, one person is permitted to remain with the patient to help him/her to drink and eat. Day and night supervisory shifts must be organized and simple treatment protocols, which are easy to implement, must be set up. The patient should be rehydrated orally if his clinical state is not alarming (at this point, the solution recommended by WHO is used) or, if dehydration is very serious or if repeated vomiting prohibits the oral route, the parenteral route with Ringer lactate should be used. Rehydration quantities to be perfused should be calculated depending on clinical signs and the quantity of liquid lost. It is not unheard-of to perfuse a very dehydrated patient with about 10 L of rehydration solution during the first 24 h. With antibiotherapy, the duration of the symptoms can be shortened for patients presenting with a significant loss of liquids [9]. Because *V. cholerae* isolates can be resistant, some samples must be sent to a laboratory to test for their sensitivity to antibiotics. Bacteriological exams need not be done for each patient. It is thus possible to have a CTC without a built-in laboratory. Nevertheless, CTCs are not light structures, and it is impossible to set one up in each cholera-infected village or neighborhood. Oral rehydration stations can be set up in those places that are far from CTCs. These are light structures, manned by a nurse, whose main role is to differentiate between those who need only oral rehydration and can be treated at the station, and those who need perfusions and therefore must be referred to a CTC. These stations can be set up as the epidemic spreads; they can relieve the pressure on the CTCs and can curb cholera complications by giving first aid. With well-coordinated access to CTCs and the consequent improvement in care, the lethality of cholera can be considerably reduced, that is, to below the threshold of 1%, as opposed to the 10% of the worst situations. This can be done by offering free care, by informing patients of the importance of getting care as quickly as possible, and by setting up as many CTCs as possible within reasonable distances from the foci of the epidemic.

Because there are asymptomatic and pauci-symptomatic carriers, even the early treatment of cases cannot stem an epidemic. The most efficient method for combating cholera is to improve access to potable water. This principal is now usually applied in refugee camps. The provision of 15–20 L of potable water per day, per person as quickly as possible has now become the objective to avoid the outbreak of a large-scale epidemic such as the one in Goma in 1994. Cholera, however, is not just specific to refugee camps; outbreaks occur even more often in open areas, such as rural areas where access is difficult, or in the poorer sections of African cities piped water is not available to all households. In these cases, *a priori*, many aid program agendas do not include water distribution. They say that it is a development problem, and development would take too long, considering the urgency inherent in an epidemic. Actually, as the two examples at the

end of this chapter will illustrate, epidemics in the field can take a long period of time to spread, more than a year, and in this case, improvements in water supply networks are possible. Experience acquired from natural catastrophe management shows that provisional water stations, made available during a critical period, can be set up in a few days. This can be as basic as cisterns, filled regularly by trucks.

Because the new vaccines have proved to be more efficient than the old ones, a vaccination campaign can be launched [12]. However, even the efficiency of the new vaccines is far from perfect and if a vaccination campaign is considered, it should be done in collaboration with research teams and WHO.

Another key point in the prevention of cholera epidemics is the building of latrines, or at least the creation of designated areas for defecation. This is a priority in refugee camps. In the field, however, it is very difficult to build latrines in time so that they will be operational during the epidemic. And even if that can be done, it is even more difficult to induce the people concerned to change their way of life right down to their most private behavior. Educational campaigns to try to do this are often proposed. This means describing the main symptoms of the disease, explaining why victims should seek care as soon as possible, and recommending good hygiene procedures such as frequent washing of hands and the use of good water for drinking and cooking. At first glance, holding a meeting to raise people's awareness seems simple, but it is actually one of the most delicate of missions to carry out. What can the success rate for the advisor and his/her audience be, when language, sociological and cultural barriers, just to mention a few, have to be surmounted? Can our concept of infectious diseases, with their microbes that no one in the audience has seen, be understood and accepted? And even if the speaker has succeeded in convincing those present, what about those, far greater in number, who did not come to the meeting because they had something else to do that day? Will they actually adopt the advice given by those who attended without a moment's hesitation? It must also be noted that the effectiveness of these constantly proposed sensitization meetings is hardly ever evaluated.

37.4 MAN AND CHOLERA EPIDEMICS IN THE NINETEENTH AND TWENTIETH CENTURIES

When a disease can be classified as an epidemic, and especially when it becomes a pandemic and spreads to several continents, it ceases to be just a medical issue and becomes also a political and social issue. And much more than any other infectious disease, cholera has maintained direct links to humans throughout the centuries. It has presented as many different epidemiological aspects and geographical ranges in just as many historical periods. A journey back to a few hundred years ago can show us threads that have always existed despite the diversity in place and times, and this can help us understand today's cholera.

Cholera was unknown to Western society before the nineteenth century, but there are traces in the Indian subcontinent, especially in the areas bordering the Bay of Bengal. Reference is made to deaths caused by diarrhea accompanied by clinical signs of dehydration in Hippocrates' writings and even in some Sanskrit texts [8]. In 1573, Garcia del Huerto, a Portuguese doctor, described an epidemic of cholera in the port of Goa, which had been recently colonized by the Portuguese [8]. In the seventeenth and eighteenth centuries, cholera confined itself to Asia. The first pandemic appeared in 1817. It began during the celebration of Kumbha, which is a traditional celebration held in the state of Uttar Prasdesh, in the north of India. These celebrations, attended by pilgrims from all over India, lasted for 3 months. The disease, probably carried by pilgrims from the estuary of the Ganges, was transmitted during the festivities and as the pilgrims returned to their homes, was spread throughout the entire subcontinent. The pandemic lasted until 1824 and spread eastward to China and the Philippines, southward to Madagascar, and westward to Iran and Turkey [54]. Troop movements played a major role in spreading the disease, particularly in India, Nepal, Iran, and Turkey; shipping commerce and pilgrimages to Mecca also helped. Western Europe was spared from this first pandemic, but not for long.

In 1829, the second pandemic broke out. It began in somewhat the same manner as the first, starting from the environmental reservoirs on the Bay of Bengal, but it spread even more quickly, and this time westward to the large Western cities. The wave hit England in 1831. On October 26th, the first patient died of cholera. But no one wanted to declare it. The political authorities, influenced by merchants and textile industry owners, refused to take any quarantine measures. For governmental authorities, cholera did not exist in England. Attempts to take measures to combat cholera were of course great hindered. And yet, never before had large Western cities, in particular London, been so vulnerable to waterborne diseases. It was the advent of the Industrial Era and the lack of work in the English countryside brought a great many of the rural population to the cities. Whole families moved to urban centers, living 8–10 in one room. By the mid-nineteenth century, the population of London had grown to 2.5 million. To reduce costs and to increase profits, landlords had buildings constructed that had no potable water supply system and no sewage system. Latrines were on the ground floor and those living on the upper floors frequently emptied the contents of their chamber pots from the windows into the street. Sewage was evacuated in open drains and gutters. With strong rains, the gutters overflowed and the water flooded houses and market places. Rivers, and particularly the Thames, which served as one of the sources of drinking water for the city's inhabitants, were thus directly contaminated by the city's sewage. After London, many other cities were affected and cholera spread over the whole of Europe, along the coast of the Mediterranean and, for the first time, to the most industrialized areas of America. Cholera arrived in France as late as Spring 1932. Officials and

doctors had time to plan strategies. In June 1931, a law on health checks at the borders was reactivated. In spite of these measures (let us remember that during an epidemic, many carriers of the germ are asymptomatic), cholera invaded France and ravaged the country as it did the rest of Europe. The number of deaths was estimated at 100,000, with 20,000 in Paris, which had a population of 800,000 at the time. The same causes produced the same effects: The state of hygiene in Paris was hardly better than that in London. The water in the fountains came from open-air reservoirs and from unguarded canals and rivers, which were used for shipping as well; the water in the wells came from a water table, which was only a few meters down and easily contaminated. Like London and the Thames, many Parisians got their drinking water directly from the Seine. Cholera spread also to the Americas, starting in the main East Coast ports of the United States. In New York, some doctors admitted that cholera had reached epidemic proportions, but many more, along with a certain John Pintard, contested this. Mr. Pintard, an influential banker, asked the doctors if they "*had the least idea what an announcement like that would do to business in the city?.*" Thus, as in London some years before, the presence of cholera was denied. This position is worth noting because it is one very often taken by political authorities when they are confronted with the presence of cholera in their bailiwicks. From New York and other East Coast ports, cholera began to spread from city to city. Quarantines were often instituted, but these were unsuccessful because travelers managed to enter "clean" cities despite efforts of the militia who were given the power to stop all travelers at the borders. In New York, cholera put an end to social life. Visitors were struck by the silence and unaccustomed cleanliness of the streets. Even on Broadway, passersby became such a rarity that a passing horseman became a curiosity. Tufts of grass began growing in the streets. It is interesting to note that the same disintegration of social life occurred in Philadelphia during the Spanish Flu epidemic in 1918.

In these times, the fight against cholera was rendered all the more difficult because the cause of the disease was unknown. Neither the make up of the agent responsible nor its links with water had been identified. Most doctors thought the disease was caused by inhalation of vapors or miasma. This belief only served to deepen the fears of the populace affected by the epidemic: They could not stop breathing so as not to be contaminated! The links between cholera and water were discovered only during the third pandemic which began in 1841 and whose course was almost the same as the previous one. In 1849, a London doctor named John Snow published a theory in which water was identified as the vector of cholera [54]. He explained that the disease could not be transmitted by air because the lungs were not affected by the disease. But his theory was either ignored or attacked by many doctors. It was not until the second epidemic wave of the pandemic reached London again that John Snow could demonstrate the solid basis of his theory. He plotted the cholera deaths on a map and found that they were concentrated in specific sections of the city. He then plotted what groups used which water sources and observed that the risk of death from cholera was much greater for the group which used the Broad Street pump than for those groups using other pumps. He informed the municipal authorities about his discovery. The pump in question was sealed off and the number of cholera cases began to fall rapidly (actually, according to a recent publication, the death rate had already begun to fall before the pump was sealed off) [48]. At that time, two companies supplied water to the neighborhood. One provided water that was relatively clean, but the other obtained its water downstream from the first, using water contaminated by the city drains. Since 1852, water companies had been required to filter their water before distributing it. A company engineer, however, admitted that the water was pumped directly from the Thames and distributed forthwith. In 1854, the wave of cholera killed 4500 people in that section of London.

The bacterium responsible for cholera was finally discovered by Robert Koch in 1884 during the fourth pandemic wave [38]. It was called *Vibrio comma*, because of its curved, comma-like shape. However, many years passed before Koch's work was accepted and validated by the international scientific community and before the idea that diseases could be transmitted by water was totally accepted by Western countries. At that point, the bacterium's name was changed to *V. cholerae*. Also, from the second half of the nineteenth century and on, there was considerable improvement in hygiene in large Western cities. Drains were improved, potable water supplies were made safer, and as of the beginning of the twentieth century, cholera was no longer a threat to industrialized countries. This is why the sixth pandemic, which began in 1899, did not reach a good part of Western Europe or North America. With the improvement in hygiene in the large cities, the features of cholera began to change; the target was no longer industrialized countries but rather those countries that were less developed. For about 100 years, the disease took advantage of the time lag between, on the one hand, the increase in commerce, the increase and improvement in land and sea transport and the developing international relations, and on the other hand, the poverty of the working classes, coupled with the overall ignorance and neglect of the medical and political authorities. The seventh pandemic, present for over 40 years, shows a very different geographical distribution, but has kept the same characteristics (described above), which caused the previous six.

The seventh pandemic began in the Sulawesi Archipelago in Indonesia. It was caused by the El Tor biotype, isolated in 1905 in pilgrims who died from cholera at a quarantine station located near El Tor in the Sinai Peninsula. In 1961, the epidemic invaded the archipelago and by the early 70s had passed along the Mediterranean and reached Southern Europe (Spain, Portugal, and Italy). It then invaded Black Africa, which had been spared for about a century. It hit Guinea first: Cholera was carried in by an

airplane. According to some reports, the plane came from Russia, and according to others, from Egypt or Mecca [29, 40]. In any case, while Guinea is recognized as cholera's point of departure for the invasion of West Africa, there were very few reports published about an outbreak of cholera in Conakry in July and August 1970. It would appear that the diagnosis was made rather quickly, but political authorities took exactly the same stance as their European counterparts in the nineteenth century, that is, they preferred to keep news of the epidemic under wraps as long as possible. Between August and December, cholera spread down a coastal band from Guinea to Nigeria. At this point, it could no longer be denied that cholera had arrived in Black Africa, but it was too late to stop the invasion. And it was now going into the Sahel. Already, in November, it had reached the city of Mopti (Mali), located on the interior delta of the Niger River. Given the conjunction at Mopti of a network of dirtied surface waterways, of a large regional fair, and of the passage of a number of nomads, moving their herds to winter pastures, cholera prospered in the city and then spread throughout all of the Sahel [40]. In a few months, it had reached Senegal, Mauritania to the west and Chad to the east. Guinea was not, however, the only African place of entry for cholera during the last three decades of the twentieth century; several distinct strains from the Asian reservoir have been revealed by molecular typing of isolates collected in Africa [18, 39]. In epidemic to epidemic, cholera crossed all of Africa, moving from one place to the next or metastasizing across considerable distances thanks to rapid transport. Pilgrimages, especially the one to Mecca, helped in the spread of the pandemic. African epidemics of the last few decades have often been spread because of armed conflict, which often has become chronic. These conflicts disturb the organization of care facilities, hinder the development of sanitation systems, and when a crisis flares up, thousands of people are forced to take to the road and end up packed into camps where, assisted by proximity, the risk for an epidemic becomes extremely high. The cholera epidemic in the Goma refugee camps in 1994 are an example of this. These camps were set up after the Rwandan gencocide, one of the most terrible catastrophes of the twentieth century. Nearly a million Tutsis (as well as moderate Hutus) were massacred. The fragility of the refugees and the lack of preparation time for setting up facilities (water, latrines, shelter ¨) were some of the factors, which triggered a cholera epidemic, which was so massive that its consequences, in terms of mortality and morbidity, defied measurement. Immense camps were set up hastily, but the influx of people was so great (about a million refugees) that preventive measures against an epidemic (construction of latrines, stocking potable water) could not be put in place in time. The quantity of drinking water distributed during the first few weeks of setting up the camps was 0.2 L of water per person per day [1]. The absolute minimum should have been 5 L. The water sources available to the refugees, who had to drink (it was impossible not to),

Fig. 37.2. Watering place near a refugee camp in the Goma area in 1994. On the right is an area for defecation. In the middle, people are bathing and on the left, others are washing dishes or clothes. These are optimal transmission conditions for cholera.

were very quickly and massively contaminated (Fig. 37.2). The epidemic that followed took tens of thousands of victims in just a few weeks. The victims' bodies were left at the sides of the roads, wrapped in their mats or pagnes. The same lightning-like clinical signs as described in nineteenth-century Europe appeared, causing the sudden deaths of some of the people.

Conflicts, however, were not essential for its survival; cholera continued on its path, often passing again through places it had been in several years before. In 2003, Mopti was hit again and as in 1970, the epidemic followed the course of the Niger River, spreading into Niger and then, the following year, into Chad. And as in 1970, contamination of water sources, and population movements, some connected with fishing and some with cattle-raising, helped spread the epidemic. Cholera is now considered endemic to Africa, but the term "endemic" is, here, fairly ambiguous. On a continental scale, cholera is certainly present permanently, but on a smaller scale, there are successions of epidemics, interrupted by periods of calm during which cholera disappears completely. Ecologists now speak of meta-endemic, where the pathogenic agents circulate throughout a large area in waves of successive epidemics. In some coastal regions, however, it is possible that *V. cholerae* O1 El Tor, temporarily or definitively, has found an environmental refuge in the lagoons and river estuaries. But whether or not it has found a perennial environmental reservoir, *V. cholerae* O1 El Tor, baptized in the Sinai and raised in Indonesia, is now African by adoption. Of course, it is still present in some Asian countries and in the early 90s invaded a considerable part of Latin America, leaving more than one million cases during its decade-long stay [2]; then, it disappeared. However, it is Africa that is far and away the most

affected. In 2003, only 26 cases were recorded in Latin America, 3463 in Asia and 108,067 in Africa [5]. Even though we know that all the cases were not reported, it is obvious that large-scale epidemics have not taken place in Asia or in the Americas. And one final note: *V. cholerae* O1 El Tor is no longer the sole cause of epidemics. Another *V. cholerae* has now appeared, and it comes from the Bay of Bengal. Its name is *V. cholerae* 0139 and is also known as the Bengal strain. It has been rampant in South Asia since 1992, but has been rarely isolated in other continents. It is, however, just as contagious as *V. cholerae* O1 and could one day be the cause of the eighth pandemic, especially because it can propagate in populations, which have been immunized against *V. cholerae* O1.

37.5 MAN, SOCIETY, AND CHOLERA AT THE BEGINNING OF THE TWENTY-FIRST CENTURY: OUR PERSONAL EXPERIENCE OF CHOLERA MANAGEMENT

In this, the beginning of the twenty-first century, cholera has not disappeared. It still exists in Asia, due to the emergence of new pathogenic strains from an environmental reservoir, but it is in Black Africa that it is most problematic. Now that this disease has been carefully studied and there are efficient ways of combating it, why and how can cholera continue to survive and even thrive? It is true that in Africa, and in Asia, there may be environmental reservoirs in certain coastal zones. However, their role has not been clearly defined and even if they do exist, this cannot explain why cholera is active in the very heart of the African continent, thousands of kilometers away from brackish water. To understand what is happening let us change scales and leave continents and countries to look more closely at small communities, and at the behavior of individuals and their beliefs about cholera. To do that, we will look at two examples of epidemics: one, which hit Grand Comoro in 1998, and the onset of one, which occurred in 2002 in Eastern Kasai (in the Democratic Republic of the Congo).

37.5.1 The Cholera Epidemic in Grand Comoro

Grand Comoro is the main island in the Comoros Archipelago. It is located midway between East Africa and Madagascar. The first cases were probably imported by plane from East Africa (where cholera was rife) toward the end of 1997. The epidemic first spread unnoticed during the month of January 1998 until two transferred patients in the hospital of Moroni, the capital city, died. This alerted the general public and the health authorities. As soon as the epidemic had been detected and the gravity of the problem had been recognized by the local authorities (this took several weeks), several strategies for combating the disease were activated. A committee was set up to coordinate these strategies; it was composed of staff from care centers, the health department, the Comorian Red Crescent, NGOs such as Médecins du Monde and Médecins sans Frontières, and health workers and representatives from WHO and UNICEF. The main strategy used was the one usually recommended: Health care structures were reinforced and CTCs were created, free health care was made available for those suffering from diarrhea, and information and personal hygiene awareness campaigns were organized. As these procedures have already been presented, we will not elaborate any further on them; but we will describe certain initiatives taken which were less pertinent, but which were exactly what the public wanted to hear. Most of these kinds of initiatives go unnoticed by those who come from outside the country and its culture because they are rarely spoken of in front of foreigners, who would surely disapprove. However, if you pay careful attention, sometimes you can make surprising discoveries. One of these "discoveries" was made during an assessment mission carried out for Médecins du Monde during the first months of the epidemic. When we arrived in a village which had been hit particularly hard by the epidemic, we were surprised to learn that the village doctor had decreed that it was not cholera (once again the denial of the disease), but "cholerine" that was the culprit, "cholerine" being a disease causing diarrhea, but was not as dangerous as cholera. Since "cholerine" does not exist in medical terminology, a brand-new disease could be invented, whose symptoms could be made to fit with the preconceived ideas of what the people had about an epidemic and its causes. Flies, therefore, were the vectors and udders of the goats, which wandered through and around the villages, became the reservoir. The therapeutic strategy was adapted to the people's expectations as well, and instead of distributing rehydration solutions (How could anything so banal cure anything?), antibiotics were distributed to anyone who passed within a radius of under 10 m of the sick person. Donor generosity equaled the originality of this therapy, because the antibiotics that were distributed were brought to the village by expatriate Comorians who had collected box upon box in France. Of course, as we know, antibiotics in this case are not only just ineffective (especially given that the strain was resistant to the antibiotics distributed) but also dangerous. It is interesting to note that the framework within which the disease was placed is a modern one (the notion of hygiene was linked with flies and animals wandering in the villages; Western therapy was used) and not one based on magic (sorcery, evil spirits). This can be explained by the influence of expatriate Comorians, many of whom leave the islands to work in France and return to their country for vacation and retirement. This argument had a greater impact, for how much weight could the villagers accord to the theories of their doctor and to those of the foreign volunteers who talked about the strange notion of "microbes," which are some kind of little invisible animals, which give you a disease if you do notwash your hands.

Besides the example mentioned above, the health committee discussed several ways to avoid transmission of the disease, but very few were selected. There are not many sources of drinking water in Grand Comoro. There are no rivers (water seeps down into the volcanic soil), some coastal villages have wells, but these are very few and far between; only those who lived in the area of Moroni, the capital, had

Fig. 37.3. "Marigot" or "piscine" on Grand Comoro. These watering places are at sea level; they are the main sources of water for the inhabitants of the surrounding villages. They are used for taking baths and washing dishes and can be easily contaminated. During the cholera epidemic on Grand Comoro, the inhabitants of the villages located near these "piscines" were very hard hit (in some villages, more than 10% of the population had to be hospitalized).

access to piped chlorinated water. The only other sources are watering places located close to the sea, called "piscines" or "marigots" by the Comorians (Fig. 37.3). The weight of the incoming tide on the underground water level fills them up. The water is more or less brackish and it is used for washing clothes, dishes, for washing oneself, and, once night has fallen, it is sometimes used for relieving one's self.

On the rest of the island, especially in rural, inland areas, cisterns collecting rainwater from the roofs are the only available sources of water. There are many of these (about one for every 10 inhabitants), but they can provide only a few liters of water per day per person. The cisterns can be contaminated easily, especially during the dry season, when several families take water from those, which still have some water. As we could not increase the supplies of drinking water in the villages and city sectors affected by the epidemic, we tried to improve the quality of the water available. When several patients from the same village or city sector were admitted to a CTC, a team from one of the hygiene committees specifically created for the epidemic would go to the place in question to try to find the sources of contamination. If a cistern was suspected of being contaminated (*V. cholerae* can survive in fresh water), it was decontaminated with chlorine. If the patients had used water from a collective water source, such as a "piscine," it was banned. This ban could not be enforced for a long time because of the scarcity of water on Grand Comoro. And lastly, so that uncontaminated water could remain that way, information campaigns were launched in which the people were told to treat their water at home by boiling or by chlorinating. As already discussed, latrines are often built to reduce environmental contamination. This measure was not carried out on Comoro, even though most households do not have a latrine, because the soil is volcanic and it is hard to dig into.

Besides transmission by water supplies and direct contact with the sick, there are social traditions, which can facilitate the transmission of cholera. Funeral rites, for example, played a particularly pernicious role in the spread of the first cholera epidemic, which occurred on Grand Comoro in 1975 [30]. In preparing the body of the deceased, the intestines are emptied by pressing on the abdomen. No particular precautions are taken for this, and it is done just before the meal is made which is consumed by the many funeral guests. This procedure also caused several outbreaks during the 1998 epidemic. We discussed this with the imams and it was agreed that specialized teams would take the place of the families in preparing the bodies for burial. Once they were created, the teams were trained and provided with the necessary materials. We have already seen how festivities in general, and communal meals in particular, have helped produce outbreaks of cholera throughout its history. In Comoro, communal meals frequently occur and they are part of the required stages for ritual ceremonies such as the "Grand Mariage," or "Anda." The Grand Mariage is the most important event in Grand Comorian society. It is the last step up the social ladder to the prestigious position of Notable; the honor of the family is assured. The *kombe*, the *badri*, the *zindru*, the *mbe zakaramu*, and the *tuarabu*, spaced out over the years, are the main stages of this elaborate and ostentatious ceremony that includes feasts, processions, and ceremonies with chanting and dancing. The feasts, with several hundreds of guests in attendance, generally include salads, meat, fowl, and fish with rice or manioc, curdled milk, honey, fruit, and fruit juice or soda. During a cholera epidemic, this type of festivity should be forbidden, but to forbid a Grand Mariage means destroying accession to the Nobility. It is at least worth trying to delay the ceremony or change the menu (eliminating the raw vegetables and curdled milk, which are easily contaminated by *V. cholerae*). As with the funeral rites, sometimes adjustments in customs can be negotiated, but the customs per se must be left intact.

Taken one at a time, solutions to problems such as those described above can generally be found. However, in the field, problems and obstacles can follow one after the other, and combined, can bring an antiepidemic strategy to its knees. An illustration of this occurred during the outbreak of cholera in the south of Grand Comoro. Up until early March, 1998, while cholera was running rampant some 50 km away, southern Grand Comoro had remained relatively untouched: A few sporadic cases had been reported and the hygiene committees had immediately taken action. Early in the month, a Grand Mariage feast was scheduled in Ouroveni, a small fishing village in the south of the island. Several hours before the feast was to begin, a cook was admitted for cholera to the Foumbouni hospital, located 10 km away from Ouroveni. She had come to help to prepare a Grand Mariage and lived in the north of the island in a place seriously affected by the epidemic. A male nurse with Médecins du Monde was immediately dispatched to Ouroveni, where he tried to inform the

people; he also requested that the feast be postponed. Unfortunately, not only were the stakes too high, given the importance of the ceremony, but the food was all ready. In the next few days about 10 serious cases were hospitalized, but many other patients who were not as severely infected, stayed in the village. There was a public well in Ouroveni, which had no protecting lip (Fig. 37.4). It could not be sealed off because it was the main source of water for the whole village. The water could not be treated in the well itself because, due to the porous nature of the lava rock, the well filled up and emptied each time the tide changed. Chlorine bleach was distributed and sensitization sessions were held during which instructions were given for treating the water or for boiling it before drinking. However, possibly because we had tried to get the Grand Mariage feast postponed, we received a cold reception. One morning, a sick person who had not been hospitalized contaminated the well. The well was used all that day, and that night the first cases arrived at the Foumbouni hospital. In 3 days, more than 100 people were hospitalized (Fig. 37.5). Most of them were women because they are the water-carriers, and because it was hot and the well is a good distance away, they drank some on the return trip. There were two deaths, one of whom was an adolescent, and in the next few days, the people's attitude changed radically. Ouroveni became a model for mobilization against cholera and its hygiene committee became the most highly motivated and the most active one in the Comoro Islands. But for southern Grand Comoro it was too late: After Ouroveni, other foci appeared and because they were overwhelmed by the influx of sick people, the medical teams had to drop the information and village community education sessions temporarily.

Fig. 37.4. Well in Ouroveni. This easily dirtied well is the main source of drinking water for the village of Ouroveni on Grand Comoro. In March 1998, it was contaminated by a cholera patient, causing the infection of more than 100 inhabitants of the village; they were hospitalized in the Foumbouni hospital, 10 km away.

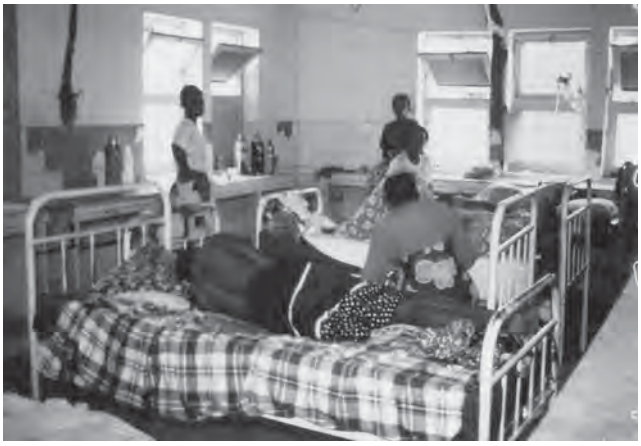

Fig. 37.5. Foumbouni Hospital. The outbreak in Ouroveni was so sudden that it was impossible to set up a CTC in time to deal with the influx of patients; they had to be put in any available place in the hospital (here, in the laboratory).

The epidemic hit the whole island, and caused the hospitalization of a total of 8000 people, 82 of whom died, and it was not until the Spring of 1999, that is, more than a year after the beginning of the epidemic, that cholera was brought under control. In the meantime, it had moved to the neighboring island of Anjouan and then on to Madagascar, where it was responsible for hundreds of additional deaths. In the survey we did in 1999, we found that almost all of the people now knew about the mode of contamination and the clinical signs of cholera. However, families had not changed their habits regarding drinking water and hygiene. We think that this is because our sensitization program proposed no alternatives to their old habits. Particularly, messages about washing more could not have much effect when there is a lack of water. No vaccination campaign was attempted in Grand Comoro. The only one carried out was on Mayotte, the one island in the archipelago, which has remained a French territory and where living conditions are better. There was no epidemic on Mayotte, but there is no proof that that was due to the vaccine.

37.5.2 Cholera Epidemic in Kasai

The recent cholera epidemic in the province of Eastern Kasai serves as an illustration of the countless problems associated with the ideas patients have about cholera as well as those problems resulting from the attitude of actors involved in the fight against cholera. It also illustrates how the behavior of a few people can sometimes help spread an epidemic. Despite its proximity to the province of Katanga (where cholera has firmly established itself), Eastern Kasai had never officially had a case of cholera. Two trains connect Lubumbashi, the

capital of Katanga, and Eastern Kasai; the railway is Kasai's lifeline. At the end of August 2002, what had been a threat of cholera became a reality. Western Katanga and the city of Lubumbashi were prey to a large-scale epidemic. The first cases were reported on September 21st in a village about 60 km northeast of Mbuji Mayi. One was a 35-year-old woman who had returned by train from Katanga several days before; she died very quickly because of profuse diarrhea accompanied by vomiting. The next day, her husband died, suffering from the same symptoms. Because no one had ever seen a case of cholera in Kasai, and these two deaths were so sudden and unexpected, the family was convinced it was a case of sorcery. A ritual was organized to identify the witch, and all the members of the families were required to attend. Actually, no demand was necessary: Everyone made haste to come to demonstrate their innocence. The ritual took place on September 23rd in Mbuji Mayi: Each person had to drink some of the water in which the clothing of the deceased had been soaking. Of the seven people who participated, five died and the other two survived thanks to the care administered at the local hospital. Funeral ceremonies for the deceased included feasts of rejoicing: The ritual was a success because the witches had been identified and were now dead. The extended family, friends and acquaintances attended all the elaborate ceremonies, and this of course helped spread the epidemic throughout the city of Mbuji Mayi. Day after day, new cases of cholera were reported and admitted to the hospitals in and around Mbuji Mayi; they were unprepared for this new disease. Besides the suspicion of sorcery, other kinds of behavior helped propagate the disease in Eastern Kasai. The outright denial of the existence of the disease was one of them. In Mbuji Mayi, there are a number of communities belonging to a sect hostile to most forms of modern life. Several members died of cholera because they were hidden in one of the sect's churches where a guru chanted over them. Only after the death of one of the gurus' wives, followed by his own, could the members of the sect be persuaded to go to a CTC.

Just as in the Comoro Islands, plans to improve the sanitation of Mbuji Mayi were discussed. However, even though everyone seemed to be convinced that improvement measures needed to be maintained from beginning to end of the crisis, in the field no one carried this out. The link between cholera and the water supply had been made obvious: In Malemba Nkulu (Katanga, September 2002) and in Mbuji Mayi (April 2004), fresh outbreaks of cholera were observed one week after the potable water supply was cut off. Unfortunately, in Mbuji Mayi, during the 2-year period when serious outbreaks were intermittent, no measures were taken to improve access to potable water. Chlorination of the drinking water containers for each house in the sectors where cases had been reported was instituted only in April 2004. A map of the sectors with reported cases was made with the collaboration of teams from the national Red Cross, who had a lot of volunteer staff in the city. The volunteers were paired and each pair was assigned one section of the city. Their job was to go to each household in the block and chlorinate each drinking water container. This was repeated steadily and the result was a sudden, significant decline in new cases of cholera. However, this outbreak had been so quickly (perhaps too quickly) throttled that it was treated as a false alarm by those who lived far away from Mbuji Mayi, and there was questioning as to whether or not there really were cholera cases. And just as in the Comoro Islands, plans that people use latrines were discussed and then abandoned, but in Mbuji Mayi, the nonconstruction of latrines was not connected with digging problems or with the lack of funds. It was connected with the wealth in the *soil*. A latrine dug in Mbuji Mayi means possibly one less diamond mine, because a good part of the city is located in a diamantiferous zone. For the inhabitants, building latrines means an imagined loss evaluated at hundreds of thousands of dollars. Sensitization campaigns were designed to ask the people to avoid at risk behavior. However, changes in behavior are difficult to achieve and the changes do not occur overnight. Results, in terms of a decrease in incidence of the disease, were slow in coming. Sensitization messages such as "Wash your hands often and heat up your food" were quickly reduced to simple slogans and had practically no influence on behavior.

Kasai also provides an illustration of how patient care procedures can cause people to delay their hospitalization so as to avoid humiliation and social disgrace. In general, the CTCs which were set up in Kasai had three sections: One for monitoring benign cases and diagnosing cases, one for the treatment of moderately dehydrated cases and one for severe cases. People were separated based on the severity of cases but not based on sex. Both men and women, therefore, were placed in the same room if they presented with the same clinical signs and the same degree of severity (Fig. 37.6). For the sick, going to a CTC meant "*saving your life and in exchange you lose your right to privacy and therefore your honor.*" In Kasai, as in many African communities, an invasion of the privacy of a person is also an attack on personal honor, especially if the person is an "Old One." Taboos connected with nudity are such that, when men, women, and children are placed in the same room, one beside the other, almost naked, on a perforated mattress where each one must "do everything," they experience extreme humiliation. In Mbuji Mayi, a tribal chief who was sick from cholera refused to go to a CTC until the Congolese CTC workers agreed to build him a shelter next to the CTC. Not all the patients had a chief's position of power, and most of the time, in despair, they had to accept a therapy that saved their lives but completely humiliated them at the same time. This explains why, in areas that have experienced it, cholera can be called "the discourteous disease." Dishonor persists after the cure, stigmatizing the patients who stayed in a CTC. For many, their entourage will consider them contagious and they can remain outcasts for a long time. Some are called "the entrance to misfortune," and will always be suspect because,

Fig. 37.6. Center for the treatment of cholera in Kasai. Note that both men (on the right) and women (on the left) are hospitalized in the same tent. Just a pagne covers their nude bodies.

according to local beliefs, "*you don't get a disease like that for no reason*."

It is interesting to note that despite the fact that the NGO teams were composed of Africans and Europeans, questions about cultural differences and behavior and their related potential problems had never come up: Medical techniques were the only criteria used when setting up the care system in Kasai. Actually there was such an imbalance in the decision-making power of the local staff and the expatriate volunteers within the humanitarian organizations that some expatriates began to lord it over the others, dictating everything and rejecting anything that contradicted what they had said. Sometimes the same problem arose in the coordination of the medical teams. When there was no good coordination, members of each side chose what to do on their own, proposing policies and strategies that were completely contradictory to the others. Each side developed and reported their own statistics, which led to terminologies such as "MSF data," "WHO data," and "Ministry of Health data."

Coordination problems are so difficult to solve because those involved do not have the same objectives. The local personnel, health authorities, expatriate volunteers, and experts from the United Nations: None of these groups see a cholera epidemic in the same way. For the local personnel, whether they are government employees or not, a cholera epidemic may sometimes be seen as a windfall because it brings in agencies from outside and means bonuses worth 10 times more than their regular salary. Thus, in Malemba Nkulu (Katanga) a CTC nurse was heard to say, "*I hope this epidemic goes on until I finish building my house*." It is therefore tempting to make projects last as long as possible by, for example, overestimating the number of cases, falsifying data or using an exaggerated definition of sensitivity for cases. In Mbuji Mayi, some health workers managed to buy themselves motorcycles (the prices ranged from $1000 to $2500) by selling packets of Ringer lactate at exorbitant prices ($25–$50). At the political level, the outbreak of an epidemic can also be used to get even with a civil servant or get rid of a no-longer-wanted NGO. Thus, once its existence is recognized in a given area, the response to cholera is ambiguous, starting at the bottom rung of the public health ladder and going up to the ministerial level.

37.5.3 Cholera, Media, and Humanitarian Agencies

Does the same phenomenon exist for international NGOs? It is not our intent to insult the courage and sacrifice of all the humanitarian volunteers by saying that they would personally benefit from a cholera epidemic by wishing it would continue longer. For the most part, volunteers are paid at a much lower rate than if they stayed home and worked in their own countries. However, if we look at the situation from the point of view of humanitarian agencies, we must admit that an outbreak, particularly when it is a cholera epidemic, can be useful for mobilizing public opinion. For Western society, cholera no longer generates the irrational fear that it did during the nineteenth century. Yes, it is still a scourge, but everyone is now convinced that it cannot happen to us now; it happens only to the "other people," the "other people" being those who live in those faraway places called "developing countries." After terrorizing the industrialized countries, cholera has now become the symbol of the world's misery and thus a tool for mobilizing public compassion. And at this level, it is not so much whether the methods for combating the epidemic are efficient or not, but how to respond to the expectations of the donor. The donors themselves, who are affected more by the dramatic urgency of the request than by the complex meanderings of development, first lend their support to life-saving acts in which "French doctors," thanks to their perfusions, save thousands of men's, women's, and even better, children's, lives. There is, therefore, the phenomenon of a, in the main, satisfying support of patients who are victims of an epidemic outbreak, linked with a lack of interest for anything that is not spectacular. The instability of public pressure forces early demobilization of action after a few weeks and the epidemic then switches to a chronic phase. It is as if firefighters, in the case of forest fires, first intervene only in cases of large-scale fires and second, withdraw soon afterward, leaving the area still smoking**,** with a real potential for starting new fires. In the Comoro Islands and in Kasai, the demobilization of the actors from outside always came too soon. Finances dried up and the people and their leaders lost interest in a disease that they were gradually getting used to living with. When the basic elements of the fight depend on voluntarism and volunteers, there can be no hope for the sustained, costly, and tiring action necessary to get the epidemic completely under control. This inconsistency is illustrated yet again when we look at some recent events. The fear that is induced by reports of cholera epidemics (whether or not there is actually a risk) produces a significant surge in public generosity. This happens after natural catastrophes (earthquakes, volcano eruptions, and tsunamis, all of which come under the heading of geophysical catastrophes). The level of generosity increases especially when there

is a high number of sudden deaths. The risk of a cholera epidemic was evoked after the earthquake in Bam in December 2003, and then again 1 year later when the tsunami in the Indian Ocean occurred [46, 57]. The media took up the chorus of the alarmist forecasts of a few experts who predicted tens of thousands of deaths because of epidemics, with cholera at the top of the list. In fact, not one case of cholera was identified after the tsunami, nor was there one after the earthquake in Bam. When the medical reports and articles on the geophysical catastrophes from the last 20 years were researched in detail, and when the available databanks on epidemics were reviewed, it is clear that no cholera epidemic broke out under these conditions. It would appear that the risk of an epidemic following a natural catastrophe is practically zero [20]. Out of the 600 geophysical catastrophes, which were identified over the last 20 years, only one was reported. It was a measles epidemic, which broke out in a displaced persons camp, which was created following a volcanic eruption in the Philippines [44]. In the end, it is not whether a cholera epidemic has actually been identified, but that the risk of one, whether real or imagined, should be announced to the public. All of this would be of little importance if cholera were not the main target. Indeed, all this mediatization about cholera epidemics, which never occur is nothing but a decoy, and the resources engaged to fight cholera where it is not a threat are not used to fight it where it is present or a real threat.

37.6. CONCLUSION

As the short tale demonstrated in the introduction of this chapter, cholera is not just an infectious disease caused by a microbe, which is pathogenic for humans: It, like the plague, has played a role in the history of mankind. What gives cholera its power is neither contagiousness (it is not as contagious as the flu), nor virulence (most patients are asymptomatic). No, its power and its continued existence despite technical progress are due to its ability to take advantage of the inconsistencies in modern human society. The ignorance of the masses and the obscurantism, which surrounds it, understandable in the nineteenth century when its causes were unknown, are still today the prime movers of its continued existence. Witness the conjuring practices (as in Kasai) and the fanciful explanations (as in the Comoro Islands). The greed of the most powerfully placed and the lowliest open the way for cholera, forcing the poorest to stay in slums, making public health measures difficult to carry out by delaying the declaration of an epidemic as long is possible. The increase in power of generous and well-informed humanitarian organizations should have brought the deathblow to this scourge and confined it to a few lagoon areas where it could be carefully monitored. Cholera has escaped, aided by the mediatization of selected events, which has an increasingly perverse control over the financial manna, which comes from the viewing public. Cholera presents with varying aspects, which are sometimes contradictory and, which allow it to have the success it has today. It is a microbe which originated in a singular coastal biotope and yet can invade cities which are thousands of kilometers from the sea; it is a fear-ridden disease for patients and yet can be a blessing for the staff of forsaken health centers in the heart of Africa; it is a disease linked to both commerce and war. Even though it has never been reported after earthquakes or tsunamis, the general public and politicians believe it to be a usual complication of natural catastrophes. In the end, our powerlessness before this scourge is perhaps not a question of lack of funds and equipment: The disease is vulnerable and some epidemics have been brought under control with fairly limited means. Our powerlessness is more likely linked to the lag between the perceptions of the actors involved in the fight: from the nurse in a dispensary in the African bush to the general public and the policy makers of the West, including the scientists, the NGO volunteers, the civil servants and the media. All these points of view must be integrated into an interdisciplinary approach: something, which is cruelly lacking today. The problem raised here is not specific to cholera. The ideas presented throughout this encyclopedia embody a movement toward this interdisciplinary approach. Would that these ideas lead, in the interest of the patients, to many others.

TABLE 37.1 Very Simplified Classification of the Main Bacteria in Human and Veterinary Medicine

- Bacilli
  - Gram negative
    - Entero-bacteriaceae *Escherichia*, *Shigella*, *Salmonella* (including *S. typhi* causing typhoid fever), *Proteus*, *Yersinia* (including *Y. pestis* causing plague), *Enterobacter*, *Serratia*
    - Non Entero-bacteriaceae *Vibrio* (including cholera-causing *V. cholerae*), *Pseudomonas*, *Acinetobacter*, *Campylobacter*, *Helicobacter*, *Bordetella* (including *B. pertussis* which causes whooping cough), *Haemophilus*, *Legionella*
  - Gram positive
    - *Listeria*, *Corynebacterium*, *Clostridium*
- Cocci
  - Gram negative
    - *Neisseria* (including *N. meningitidis* causing *meningitidis*), *Branhamella*
  - Gram positive
    - Packets of cells seen on Gram stain
      - *Staphylococcus*
    - Pairs or chains of cells on Gram stain
      - *Streptococcus*
- Other bacteria
  - Small intracellular bacteria *Chlamydia*, *Rickettsia*
  - Spiral organisms *Treponema* (including *T. pallidum* which causes syphilis), *Borrellia*
  - Small pleomorphic without rigid wall
    - *Mycoplasma*
  - Acid-alcohol fast bacillus (using Ziehl–Neelsen stain)
    - *Mycobacterium* (including *M. tuberculosis* and *M. bovis* which causes tuberculosis, and *M. leprae* which causes leprosy)

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CHAPTER 38

Infectious Diseases: Market of the Future?

Jan Verhoef and Ad Fluit

Eijkman-Winkler Center for Medical Microbiology, Infectious Diseases & Inflammation, University Medical Center Utrecht, Heidelberglaan 100, Rm G 04.614, 3584 CX Utrecht, The Netherlands

38.1 INTRODUCTION

We are facing a second revolution in medicine and a third revolution is looming. One hundred years ago, the world was on the brink of the first revolution. Then, scientists like Pasteur, Koch, Ehrlich, Röntgen, and their students set the stage for a revolution: first they proved that microorganisms could cause infections; that infections could be prevented; that pathologic processes could be visualized; and that malignant processes could be recognized.

The developments of the nineteenth century led to mass prevention of infections, to better surgical techniques, and to better diagnostic possibilities. In fact, they influenced all medical disciplines. Expectations were raised when Fleming discovered penicillin in 1929: The medical world was considered capable of combating many diseases and increasing human life expectancy. Forty years later, in 1969, it was thought that the book on infectious diseases could be closed. The Inspector General actually told the President of the United States that investments in the treatment of infectious diseases should be given low priority because of available antibiotics and vaccines. Even though infectious diseases had always been major killers worldwide, the western world was positive that it had won the battle against microbes [6].

Nothing was farther from the truth, infectious diseases have been (and still are) emerging or reemerging everywhere. Legionnaire's disease, for example, was discovered in 1976 and the first patients with AIDS were described in 1981—no one could have foreseen at that time that AIDS would become a global calamity [22]. Some forms of rheumatic diseases are now known to be caused by microbes; gastric ulcer, a very painful disease that used to be treated by surgeons, is no longer a surgical procedure because of the discovery that this type of ulcer is caused by bacteria.

In addition to the naturally caused infectious diseases, man is now also facing bioterrorism: The use of microorganisms to deliberately disseminate disease to do harm to large groups of people as an act to terrorize, as was witnessed in the United States in 2002 when Anthrax was spread in posted envelopes.

The emergence of bacterial and viral resistance to antimicrobial drugs is another great threat to mankind. Not only many hospital bacteria but also bacteria in the community have become resistant to antibiotics over the past years. Unless adequate measures are taken we could soon find ourselves again in an era without adequate drugs. There is, therefore, a desperate need for new antibiotics.

The situation is worsened by the fact that rapid and adequate tests for the diagnosis of infectious diseases are lacking. All of these developments are putting infectious diseases back in the medical spotlight [11].

Thus, there are three major interrelated problems in the management of infectious diseases in the developed world that have put infections back in the priority list of physicians. They are

- the increased complexity of patients with infections (unexpected resistance problems, exotic infections in immunocompromised patients, etc.);
- a lack of new antibiotics that can be added to our arsenal against resistant microorganisms;
- a lack of rapid, adequate tests to diagnose infections.

In the late 1970s and 1980s, the medical world was extremely optimistic that in the not too distant future it would be able to diagnose infections almost instantaneously, that the metier of the microbiologist would soon change completely, and that genomic research would rapidly deliver completely new antimicrobials. This was a wishful thinking as in 2005 we have still not witnessed these dramatic changes.

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

The laboratory tests necessary in the diagnosis of infections still takes 2–4 days, and the need for new antibiotics is ever increasing.

Despite this sober mood, we are on the brink of a second revolution in medicine. This revolution will not be driven by the results of biomedical research, but by information technology. The availability of expert software programs will lead to better patient-care. The physician is guided to the most adequate diagnostic tests, to the best patient-care, and to the use of the most appropriate antibiotic. Ultimately, although later than anticipated, the results of molecular biology, genomics, and proteomics will start a third revolution. Within 20 years these two revolutions should change the face of the discipline of infectious diseases.

Seen in this perspective, infectious diseases should support a very interesting niche in the market with many opportunities.

38.2 THE INFORMATION AND COMMUNICATION TECHNOLOGY REVOLUTION

Imagine how a patient will be managed in the future. When an individual wishes to consult a doctor, he/she will first be asked a number of questions, often *via* the Internet, before actually seeing the primary care doctor and then will be asked to undergo a number of basic tests. When the doctor inputs his/her preliminary findings, the computer will dictate the avenues to follow and which tests should be requested from the laboratory, radiology, and among others. It is this expert system that will come up with a presumptive diagnosis, not the doctor.

Before seeing the specialist the patient will be referred to the Department of Diagnostic Medicine, which includes medicinal chemistry, pathology, immunology, microbiology and radiology (imaging), ECG, and other departments (Fig. 38.1). The results obtained in this department will be aggregated and committed to a rigid quality control system. All irrelevant information will be discarded, or at least put in a separate database. The specialist will receive only relevant results from the Department of Diagnostic Medicine. The computer, whose programs will be constantly updated *via* the Internet, will now confirm the working diagnosis, change the diagnosis, or order new tests. When required, the computer will also dictate the treatment regimen.

38.3 INTERNET CHANGING THE HEALTH CARE DELIVERY LANDSCAPE

Much of what has been available in print format is out-of-date or hard to find. The Internet is emerging as a unique communication and distribution channel for health information worldwide. The rapidly expanding content and commerce offerings, coupled with the relatively low barriers to enter the World Wide Web, contribute to massive Internet growth. Professionals are seeking "just-in-time" information that the Internet provides. In fact, enormous amounts of digital information are already available to health professionals today.

The expert systems available on Internet offer a wealth of information. However, this information is not useful unless it is distilled by experts, it is up-to-date, and presented in a comprehensive, current, and easy-to-use format. Because physicians and other health professionals are working under increasing time pressures and struggling to stay abreast of current medical practices, there is a need for comprehensive, up-to-date expert systems [1].

Personal digital assistants (PDAs) are capable of changing health care even more in the future. Most hand-held PDAs run on the mobile operating systems of either Palm OS (PalmSource Inc, Sunnyvale, CA, USA) or Microsoft Windows (Microsoft Corp, Redmond, WA, USA) that allow the installation of third-party software applications. An example of commonly used medical software is shown in Figure 38.2.

Furthermore, some Palm OS or Windows mobile-based PDAs have a Java (Sun Microsystems, Santa Carla, CA, USA) runtime that allows the use of platform-independent, Java-based

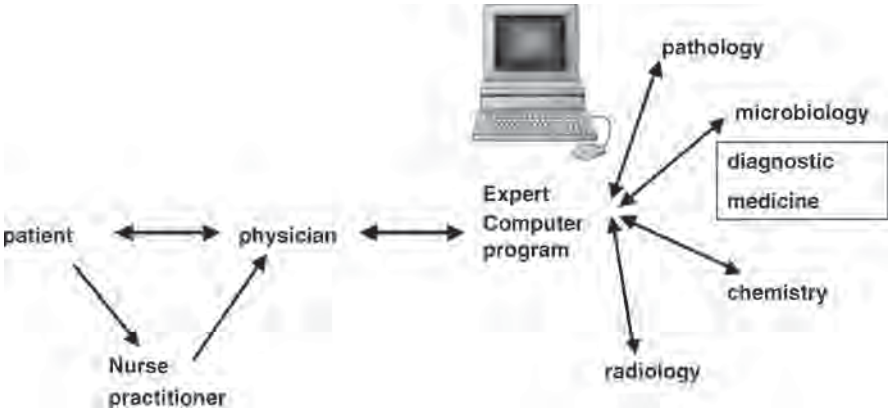

Fig. 38.1. Flow chart depicting the route a patient takes before seeing a specialist.

Fig. 38.2. The screen of a hand-held device depicting examples of commonly used medical software (from D.C. Baumgart, Lancet 2005:366:1210–22).

applications. Other platforms, such as Newton (Apple Computer, Cupertineo, CA, USA), Psion (Psion Teklogix, Mississauga, ON, Canada), BeOS (PalmSource Inc), Symbian OS (Symbian, London, UK), and Blackberry (Research in Motion, Waterloo, ON, Canada), currently have no major role in the health-care market [1].

To demonstrate the role of PDAs in the professional life of a physician, Baumgart sums up

> On a typical day, my PDA wakes up 30 minutes before I do, logs on to my notebook as well as the internet, and synchronises and updates all PDA applications. Not only are contacts, appointments, and medical references kept up-to-date in this way, but my PDA e-mail application is also programmed to retrieve exclusive e-mails such as electronic tables of contents from medical journals, alerts from the FDA medwatch system, and other resources in my e-mail inbox. On the way to work I can review, mark, and erase these e-mails. Once at the hospital my PDA reminds me of conferences, meetings and displays a to-do list for the day. When I see patients I rely on drug reference and interaction applications, institutional microbial spectra databases, medical calculators, prediction rules and specific topics in PDA editions of popular medical reference manuals. Additionally, I have many guidelines from our institution, professional organisations, and agencies, as well as pdf excerpts from journal articles stored in my memory card. I do not believe PDA versions of large medical textbooks are helpful, because they are often difficult to navigate. I am currently investigating the usefulness of the new Pubmed on Tap programme, whenever I have access to the institutional W-LAN.
>
> Our department receives a fair amount of patients with gastrointestinal cancers. Staging of uncommon cancers is easy with a TNM (tumour, node, metastasis) staging programme. I can customise and print actual chemotherapy protocols with a shareware application. An add-on to this shareware application allows me to programme and print protocols for rare cancers. At ward rounds with students, I take full advantage of the multimedia capabilities of my PDA: I can display images from my personal medical image library or other PDA reference materials, play heart murmur or lung sound recordings, and use the screen to quickly sketch something to make a teaching point. I can carry and share with students (via infrared) a self-created collection of text notes, customised to the patients we see together.
>
> In clinics where I see patients enrolled in clinical trials, I quickly enter, access, and sort essential data on spreadsheets. The spreadsheets were created with my notebook spreadsheet application, transferred to and updated on my PDA with a commercial programme. This software also helps me to review and store my presentations for lectures and talks. New versions of PDAs can also act as USB memory sticks. At the end of the day, my PDA synchronises and backs up the days data with my note book before it charges for the night [1].

Furthermore, Baumgart raises the question

> What will a medical PDA look like in 2015? It will probably be housed in a ceramic or lightweight alloy case, and hopefully be no larger but substantially lighter than current shirt-pocket-sized devices. New semiconductor technology will allow handheld computers to be equipped with processors that can handle much more work than the best desktop systems that are currently available, while consuming less power to extend battery life. Memory will no longer be an issue, because data will be mainly kept in network storage systems. Manual data entry is still a problem in current versions of PDAs. In the future, authorised, secure, logons to the PDA and data entry will be done with combined speech and fingerprint recognition by sophisticated audio hardware and a new high-resolution generation of touch-sensitive screens. Graffiti 2 (PalmSource Inc) characters will be further developed into true handwriting recognition. Speech processing will also be a reality, replacing many dictating methods currently used.
>
> Very high network speeds will provide immediate access to clinical and administration data, including imaging information such as procedural movies; three-dimensional ultrasonography; CT, MRI, or PET scans; histological slides, microbial cultures; and institutional and remote reference systems at any place and time. Medical applications will go beyond organisations and storage of information. PDAs could evolve into expert systems that access information from many sources (i.e., classic texbook style references, data from basic and clinical research and genome scans, environmental and public-health information, and results from ongoing clinical trials, match the information with the patient's medical records from current or past admissions or visits, apply prediction rules, calculate clinical equations, and integrate all the data into an overall information package for clinicians. Users will be able to obtain and share opinions on patients with colleagues and international experts with ad-hoc medical multimedia conferencing [1].

Target Market: New up-to-date expert systems for the treatment or diagnosis of infections and for infection control

targets at several categories of health professionals: physicians who specialize in infectious diseases, primary care physicians, microbiologists, infection control professionals, hospital epidemiologists, perioperative nurses, and product purchasing managers.

Market Assessment: Inappropriate antibiotic use—a worldwide concern—occurs when a physician prescribes the wrong antibiotic for a patient's illness or prescribes an antibiotic when none is indicated. This inappropriate use sometimes occurs because of the complex choices that physicians face when prescribing an antibiotic. The level of antibiotic resistance in a given geographic area, the wide choice of available antibiotics (more than 280 different antibiotics) and the confusing number of indications for which each antibiotic is approved, affect these complex choices. A study reported in 1998 in *The New England Journal of Medicine* reported savings of $18,550 per ICU patient when appropriate antibiotic therapy was prescribed according to the recommendations of a computer-assisted program for antibiotics [5]. The infection control world is dynamic, with new information regarding outbreaks, alerts, medical products, legislation, public health policies, and breaking news becoming available every hour. Access to this information as well as to regulatory actions, health and safety requirements and tools for training are critical in the day-to-day practice of an infection control professional. These specialists require current, comprehensive, and easy-to-use tools that provide information on relevant issues and enable them to be more effective and efficient in their roles as educators and leaders in infection control for their institutions. Meanwhile, international travel makes the diagnosis of infection even more complex.

Today, doctors must be aware of exotic infections and infection problems originating thousands of miles away because patients can be infected elsewhere, travel in its incubation period and show signs and symptoms of an infection to a doctor unfamiliar with the symptoms. Here, alerts *via* Internet updates could really be very helpful.

As with all Internet applications the generation of a cash flow by companies that produce expert systems is difficult to predict.

Many Internet sites use commercial or links with commercial sites as a source of income. It is clear for healthcare-related Internet sites to become successful, the content must be unequivocally independent. There is a need for standardization and quality control for data obtained through the Internet. How this is compatible with profit-making Internet application is still unknown. Are professionals willing to subscribe to independent up-to-date comprehensive information while they can obtain data (albeit not filtered) without a rigid quality control system for free?

However, the use of comprehensive up-to-the minute expert systems can lead to more appropriate antibiotic use and thus better patient-care, and likely to a decrease in resistant microbes.

38.4 THE LOOMING BIOTECH REVOLUTION

38.4.1 Resistant and Multiresistant Bacteria

As mentioned, today the treatment of infection is becoming more difficult because of the increase in antibiotic resistance and the lack of fast "point of care" diagnosis of infection. Antibiotic resistance is considered a serious problem in many hospitals, and there is a clear relationship between the number of antibiotic prescriptions and the occurrence of resistance. Treatment options for multiresistant bacteria are often very limited or even unavailable. There is a fast-growing need, therefore, for novel effective classes of antibiotics [15].

Many patients with infections are currently being treated empirically because cultures take 48 h to perform. Empiric treatment is by definition broad-spectrum-based: It must be active against many bacteria. If the causative agent is recognized within a few hours, however, directed therapy can be used. Therapy that is by definition focused on the cause of infection has a small spectrum and does not expose bacteria belonging to the normal flora to antibiotics. Consequently, the emergence of resistance will decrease. Rapid diagnostics therefore is one way to prevent resistance, to prevent hospital-acquired infections, and to provide the clinician with a fast answer to his/her diagnostic problem. When included in the hospital information system, rapid diagnosis of infection can also be a part of the decision-making process at a very early stage.

38.5 RAPID DIAGNOSIS OF INFECTION

Faced with a patient with a possible infection, the clinician collects patient's samples for the laboratory to be tested.

The basic questions posed in the diagnostic laboratory are as follows:

- What is the identity of isolated bacteria?
- Is the identified bacterium a pathogen?
- Is there a need for antimicrobials?
- What is the resistance pattern?
- Does the bacterium belong to an epidemic clone, that is, is there a risk for uncontrolled spread?

Although the clinical symptoms of the patients and the initial microscopic and macroscopic observation of clinical specimen may already suggest the cause of the infection to the expert clinical microbiologist, the identity of the microorganisms can only be obtained by the use of laboratory techniques. Most identifications, by far, are performed by (automated) phenotyping. Molecular typing assays like polymerase chain reaction (PCR)-based systems are only used for very specific bacteria.

Identification of Bacteria: The identification of bacteria is based on their culture followed by chemical/biochemical or immunological testing. The advantage of culture-based identification procedures is the relative simplicity of testing. The

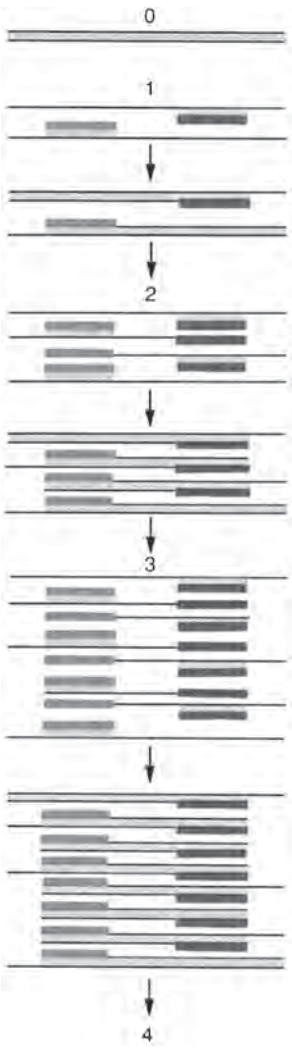

Fig. 38.3. Schematic presentation of the polymerase chain reaction. Red and blue bars indicate the primers; single line single-stranded target DNA; grey indicates double-stranded DNA.

disadvantages, however, include lack of specificity and lengthy processing times. Approximately 30% of the bacteria are identified within 48 h, some 25% within 72 h and the rest after 3–6 days. However, some bacteria cannot be cultured and therefore not be identified.

Molecular-Based Identification: Molecular-based identification methods are tests that are capable to detect genomic sequences of a microorganism. To identify the bacteria, most of these assays detect either DNA or RNA. They must first, however, amplify the DNA or RNA in order to obtain a sufficient amount for detection, for example, by PCR [7] (Fig. 38.3).

In principle, culturing is not necessary. Sufficient pure DNA or RNA, that is, DNA or RNA not contaminated by RNA or DNA from other bacterial and/or human sources, is needed. The *advantages* of molecular typing include

- the speed of analysis as direct testing from patient material may be possible;
- the possibility to study the epidemicity of certain bacteria (individual clones can be distinguished, the spread of these clones to other patients can be detected [individual fingerprinting]).

The *disadvantages* include

- the presence of PCR-inhibiting compounds in, for example, blood products and faeces;
- unsatisfactory specificity: in mixed infections or in infections where the infection site is also colonized by commensal flora, bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) and nonpathogenic methicillin-resistant *Staphylococcus epidermidis* are hard to distinguish by using PCR;
- the (current) inability to determine antibiotic resistance through existing molecular typing methodologies;
- the present databases of, for example, DNA sequences like the National Center for Biotechnology Information (NCBI), provide only limited information that is quality controlled; the common databases mostly contain information on nonpathogenic commensals.

Currently, molecular-based identification is a specific tool for identifying slow-growing bacteria for which culturing would require weeks or more (e.g., tuberculosis-causing bacteria). Numerous molecular assays, including commercial and homemade tests and a large range of targets and primers are in use today. Most of the tests have not yet been validated, however, and reproducibility between laboratories is very difficult.

38.6 LABORATORY AUTOMATION

Automation in the clinical laboratory has been an ongoing trend for many years. It began with the multichannel analyzer in the mid-1960s and advanced to the introduction of automated immunoassay systems [21], walkaway systems for microbiology analysis, and most recently automated specimen-processing and handling systems (Fig. 38.4).

The implementation of a high-level automation in many laboratories has been greatly stimulated by the shortage of technologists and technicians, particularly in the United States, coupled with increasing test volume requirements. Based on surveys conducted in 2002, for example, the vacancy rate for laboratory technologist positions is 9.5% (12% in rural facilities and 8% in urban facilities). This situation is not likely to improve in the future because of the lack of training programs: The estimated number of clinical laboratory science positions to be filled annually in the United States is about 9300 (5300 new positions and 4000 due to retirement), whereas the total number of new graduates of clinical laboratory science training programs is estimated to be about 5200. Although recent surveys indicate that the labor shortage may be improving slightly, their results may be skewed by the elimination of a number of laboratory positions that

Fig. 38.4. VITEK—automated specimen-processing and handling system (BioMerieux). See color plates.

could not be filled, causing a reduction in the reported vacancy rate even though the supply of workers has not increased substantially. Furthermore, the slight apparent improvement in the labor situation in the United States falls far short of satisfying the unmet need projected for future years, because many laboratory workers are nearing retirement age [18,23].

Another factor driving demands for automation is consolidation. According to data from the US Center for Medicare and Medicaid Services, for example, the number of hospital clinical laboratories declined by 2.9% over the 1996–2002 period. In contrast, the overall test volume, which declined 1.3% per year between 1996 and 2000 in the United States as a whole, grew about 3% in both 2000 and 2001. Based on trends quoted by clinical laboratories in 2003, the test volume is continuing to increase. Because there are fewer laboratories to handle the demand for testing, there is a need for an increased capacity in the remaining facilities. In many cases, the only practical solution is automation, providing an opportunity for suppliers of clinical laboratory automation products.

Cost savings are generally not a major factor driving decisions to adopt automation in the clinical laboratory. Although some savings can be achieved, most of the reduction in operating cost results from the reengineering and computerization of laboratory procedures to make them compatible with automation. A consensus panel discussing the topic at the 1998 Oak Ridge Conference in Raleigh, North Carolina, sponsored by the American Association for Clinical Chemistry, estimated that about 35% of the cost savings derived from laboratory automation can be attributed to process reengineering and 30% to computerization, whereas only 17% savings are attributable to the mechanical automation of specimen handling [http://www.clinchem.org, accessed in 2005].

Perhaps, a more important additional driver of the trend toward automation in the clinical laboratory is the potential for reducing errors, for example, sample misidentification. The current almost universal use of bar coding samples and secondary tubes, coupled with automated tracking of operations in the laboratory and automated reporting of results, has eliminated most of the manual entries of identifying information and other data, thus avoiding one of the key sources of error in laboratory processes.

38.7 MARKET

Test volume and the worldwide dollar volume market for conventional microbiology products have continued to grow over the past 5 years, and continued growth is projected for the future. This growth is due to a number of factors. Two of these factors are the continued increase in the incidence of infectious diseases, including sexually transmitted diseases, and the increased mobility of the world's population, resulting in the global spread of diseases that were formerly contained in isolated regions. The SARS epidemic and the West Nile virus are two recent examples of significant threats to the United States and other developed countries. There is also an increase in the incidence of mixed infections, particularly in immunocompromised patients, as well as an increase in infections with antibiotic-resistant microorganisms due to overutilization of antibiotic therapy and the widespread use of antibiotics in the food chain. According to experts from the US Centers for Disease Control and Prevention, more than 30 new microorganisms of clinical significance including *Borrelia burghdorferi*, West Nile virus, HIV, have appeared in the United States since 1967.

Another important growth factor in the microbiology sector is the increase in patient acuity that has been observed in many hospitals over the past few years. For example, although the number of discharges in the US Medicare population for infections and parasitic disease remained relatively constant between 1995 and 2000, the average length of stay in the intensive care unit as well as the number of intensive care days increased substantially. These data indicate that hospitalized patients with infectious disease are sicker but are discharged from the hospital more quickly. This phenomenon typically requires more testing to be performed over a shorter period of time.

TABLE 38.1. Indicative Market Size on Bacterial Identifications in Clinical Microbiology

Country/Continent	Inhabitants	Annual Turnover for Bacterial Tests	Turnover	Annual Bacterial Identifications	Turnover
Netherlands	16,000,000	1,500,000	€37,500,000	525,000	‡5,250,000
EU	400,000,000	37,500,000	€937,500,000	13,125,000	‡131,250,000
USA	280,000,000	26,250,000	€656,250,000	9,187,500	‡91,875,000
Japan	130,000,000	12,187,500	€304,687,500	4,265,625	‡42,656,250
Canada	30,000,000	2,812,500	€70,312,500	984,375	‡9,843,750
Total		78,750,000	€2,006,250,000	27,562,500	‡275,625,000

The indicative market size in terms of annual turnover for bacterial tests in the five major countries is approximately ‡2 billion; for the bacterial identifications this is some ‡300 million.

In nonhospital settings, such as nursing homes and long-term care facilities, and in the home health care arena, there is a high rate of infection as well as a large and growing patient population that is causing an increased demand for microbiology testing. According to Strausbaugh, of the Portland VA Medical Center (Portland, Oregon, USA), who discussed infectious disease testing in long-term care at the 2002 Annual Meeting of the American Society for Microbiology, there are 1.6–3.8 million cases of infection in long-term care facilities in the United States alone each year, which account for 26–50% of the transfers from long-term facilities to hospitals. In addition, the number of nursing home residents in the United States is expected to increase from the circa 2 million now to 5 million by 2030 [20].

The number of bacterial cultures and the number of bacterial identifications are summarized in Table 38.1. The estimations are based on the actual numbers of tests performed in the major academic centers in The Netherlands and an estimation of their numbers in other district and medical laboratories. The numbers for the listed continents are extrapolations of data based on population size.

38.8 FUTURE MARKET

38.8.1 Molecular Diagnostics

Molecular diagnostics is the most rapidly growing segment of the in vitro diagnostics market and is expected to continue to be one of the highest growth segments over the next 5–7 years. This field has expanded from its initial focus on infectious disease detection to include applications such as viral load testing, viral genotyping, pharmacogenetic testing, cancer screening and diagnosis, and genetic testing for hereditary diseases. A new major segment of the market of infectious disease-screening tests performed on blood products has also emerged within the past 2 years. This is based on the ability of molecular methods to directly detect an infectious agent prior to the development of a host immune response.

Molecular diagnosis is not based on the culture of microorganisms but on the detection of molecules belonging to these microbes. Usually microorganisms are detected and identified by DNA amplification methods, for example, by PCR. However, with the development of DNA chips, microarray technology (Fig. 38.6) may overtake simple amplification-based methods. The DNA chip technology simultaneously allows for the generation of a genetic fingerprint of the pathogen (genomics). With the development of Surface-Enhanced Laser Desorption/Ionisation-Time-of-Flight (SELDI-TOF) mass spectrometry identification of bacteria by their protein profiles becomes possible [proteomics].

BOX 38.1 VIRAL LOAD

Viral load (the number of viral particles) testing has revolutionized the management of AIDS by allowing physicians to monitor the results of drug therapy in order to detect the development of drug resistance at a much earlier stage than was possible with prior methods. Further, new disease-related genes are being identified on an almost daily basis, and information from the human genome project is providing clinicians with new insights not only in the pathogen but also in determining the patient's response to an infectious agent. It may well be that genomic and proteomic approaches may predict the outcome.

In effect, information on genetics will serve as an addition to the patient's history. A considerable amount of development of the informatics infrastructure in diagnostics, however, will be required before such an approach can become widely utilized [19].

BOX 38.2 – POINT-OF-CARE TESTING

Point-of-care testing, including testing in alternate sites in the hospital as well as testing performed in physician offices and clinics, other remote sites, and by patients themselves, remains an area of growth within the industry. At present, however, most of the growth is attributable to the continued expansion of

the market for blood glucose self-testing. Blood glucose monitoring is, in turn, expanding because of the increasing prevalence of diabetes in developed countries and the clear evidence of the benefits of a tighter control of blood glucose in preventing the long-term complications of diabetes. Although growth in the market for blood glucose self-testing products is gradually slowing as the market matures, new developments such as less invasive testing and alternative site sampling along with recent studies showing the major benefits of tight glucose control in the intensive care setting are helping to sustain a growth rate well above that of the overall diagnostics market. Other segments of the POC testing market are expected to exhibit strong growth over the forecast period and include POC cardiac marker testing for the rapid diagnosis and triage of chest pain patients; coagulating monitoring, and sepsis detection.

38.8.2 Microarrays and Lab-on-a-Chip Devices (Fig. 38.5)

Microarray technology, which allows analysis of potentially thousands of disease markers simultaneously using microliter sample volumes, has already entered the clinical arena and is expected to have a growing impact on the diagnostics market in the future.

Because of the relative immaturity of the existing devices and the lack of widespread need for analyzing large numbers of targets in a single assay, most experts do not expect this technology to have a major impact for at least 5–10 years.

Lab-on-a-chip devices are at an even earlier stage of development, and questions remain about the ability of such devices to meet the requirements of clinical testing. The potential exists, however, for reducing the volume of sample and reagent needed to perform a test by 10- or even 100-fold, significantly reducing cost and space requirements.

Miniaturized analytical systems can also in principle incorporate redundancy, multiple levels of self-testing and quality control, and backup capabilities in order to increase the reliability of test results and minimize maintenance requirements. More rapid tests are potentially possible with miniaturized devices because the necessary diffusion lengths of the molecules are shortened, leading to more rapid reaction times, and the time required for transfer of samples and reagents within the test system is reduced. Miniaturized IVD systems that integrate and control complex functions on a chip to automate the entire testing process are likely to be especially useful in the less-developed regions of the world, where the availability of sophisticated laboratory services is lacking today.

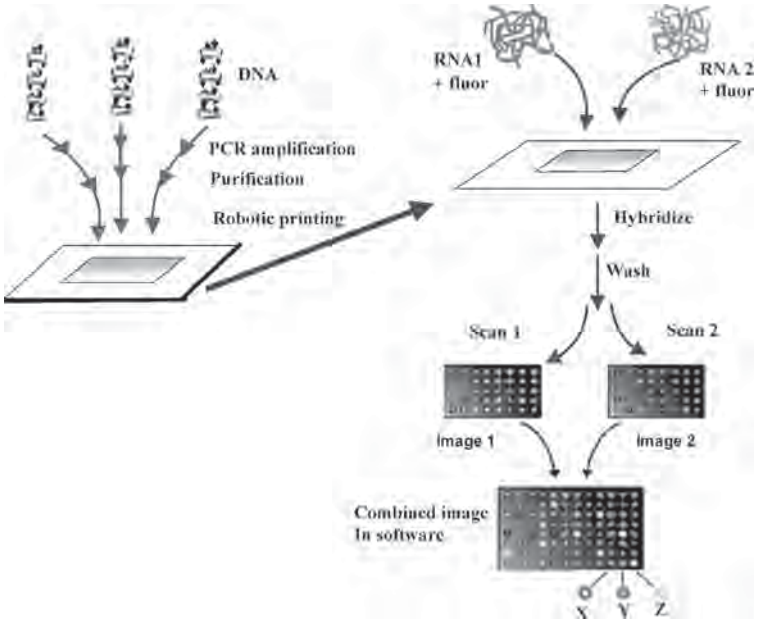

Fig. 38.5. Schematic presentation of microarray technology. Specific DNA fragments obtained by PCR amplification are printed on a slide after purification. The slide is then hybridized with probe. The probe can be either RNA or DNA. The reference probe is labeled with a red fluorescent dye and the sample with a green fluorescent dye. In red spots only the reference RNA or DNA is binding, in green spots only sample RNA or DNA is binding, and in yellow spots both are binding. This allows conclusions about the presence or absence of RNA or DNA compared to the reference isolate. See color plates.

38.9 ANTICIPATED DEVELOPMENTS

38.9.1 Real-Time PCR

Although not an identification tool *per se*, real-time PCR allows for a much faster performance of PCR (2 h instead of 6 h) and provides an ongoing insight into the quantity of microbial DNA. As such, it is possible to obtain substantially more certainty in one run [10].

38.9.2 Raman Spectroscopy [14]

Research is ongoing for the development of a fast, nondestructive, and reliable identification method using micro-Raman spectroscopy. This technique allows the irradiation of any component larger than 1 μm^2 and thus the collection of its characteristic Raman spectrum, free of interference from surrounding media. Raman spectroscopy yields information, for example, on the molecular composition of the entire bacterial cell (fatty acids, carbohydrates, proteins, nucleic acids) [14]. However, Raman spectroscopy requires the culture of the isolated bacterium. It delivers a spectrum representing the internal composition of the bacterium and is, as such, dependent on the culturing conditions. Moreover, the interpretation of the spectrum is subjective and depends on the database delivered by the manufacturer of the equipment. It is debatable whether this database is reliable and up-to-date. Raman spectroscopy has potential as a new method for bacterial identification in niche applications.

38.9.3 Whole Genome Sequencing

In principle, whole genome sequencing involves the sequencing of the entire genome. Unfortunately, this technique is not only laborious and time-consuming but also it allows for the analysis of only one strain at a time.

38.9.4 DNA Chip Technology

A crucial development in the field of identification in the coming 5–10 years will be in comparative genomics using DNA chip technology. Like many of the other technologies, the use of DNA chips is based on the comparison of reference data from more or less reliable databases. The DNA microarrays or biochips contain a surface on which multiple capture probes consisting of DNA sequences of biomarkers specific for certain bacterial species or resistance mechanisms are covalently linked, each one specific for the binding and detection of one DNA target. The advantage of the chips is that they allow the detection of multiple genomic sequences in a single assay rather than the detection of each one separately. It is anticipated that this technology will provide possibilities for refined, broadly applicable and cost-effective identification, based on genome sequences. Contamination of bacterial DNA with small amounts of, for example, human DNA forms a substantial problem in daily practice.

One example of such a chip is the Staphychip®. This DNA chip allows the molecular identification of the *Staphylococcus* genus and its species using only one duplex PCR amplification. Amplicons are hybridized on a set of genomic probes linked on the chips:

- a consensus genus-specific probe allows the detection of staphylococci in clinical samples of the environment, regardless of the species;
- various species-specific probes allow the specific identification of five of the most common staphylococcal species in human pathology (*S. aureus*, *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. saprophyticus*);
- an *mecA*-specific probe allows the detection of the main staphylococcal resistance determinant responsible for cross-resistance toward all beta-lactams antibiotic among the staphylococcal species.

Clinical relevance of this device is currently assessed in a range of applications [3].

38.10 NOVEL ANTIBIOTICS

The treatment of patients with infections has always been a lucrative market for the pharmaceutical industry [2].

In 2000, the worldwide sales for existing antibiotics were about $25 billion and is forecasted to exceed $40 billion by 2010. North America is by far the single largest market and also the fastest growing, with annual sales of $11.1 billion for antimicrobial agents (2000) and a growth over the previous 1-year period of 11% based on prescription sales at retail pharmacies. Europe is the second largest market. Germany, France, Italy, UK, and Spain have retail sales of about $4–5 billion. Japan is third at $5.5 billion in sales, including both retail and hospital prescriptions. The market is expected to grow 5–10% on a yearly basis.

One of the underlying factors that determine growth are the growing number of infections due to the increase in immunocompromised patients (the elderly), patients undergoing aggressive medical intervention (implants, organ transplantation, chemotherapy, etc.) and the increasing number of infections caused by multiresistant microbes. Because historically, *beta-lactam* (penicillins, cephalosporins) antibiotics were the first antibiotics to be discovered, they have always dominated the market. Drug resistance has become a major problem associated with these antibiotics. Because of the rapid increase in infections caused by (multi) resistant bacteria, new classes of antibiotics are needed [13,16]. So far, most "new" antibiotics are in fact often variations on common themes, against which cross-resistance may relatively easily occur.

A successful antibiotic must satisfy a large number of criteria. It should be able to cross bacterial cell membranes in sufficient concentrations, not be a target for modification by bacterial enzymes or for efflux pumps among others.

Combinatorial chemistry is now most frequently used to build a new diversity that is based on antibiotics in clinical use, including tetracyclines, macrolides, and oxazolidinones.

Other examples are tigecycline and faropenem. Analogues may have an improved therapeutic value, and financial costs may be less than for the development and marketing of completely new antibiotics. Finally, there is another economic motive to develop analogs as they may enlarge or circumvent the patent on the original antibiotic.

However, resistance to new analoges of current antibiotics may occur fast due to cross-resistance with the existing antibiotics.

38.11 NEW CLASSES OF ANTIBIOTICS

New classes of antibiotics can be discovered using two different approaches. One approach starts with the identification of new bacterial targets, for example, through comparative genomics. These are genes or gene products that are present in certain bacteria but not in eukaeryotic cells (e.g., human cells) and are no targets for known antimicrobials. These targets could be used to find compounds with a unique spectrum of activity and without effect on host cells. The other possibility tries to identify new lead compounds and their corresponding targets through functional assays. Lead compounds may be obtained from natural sources or libraries of small molecules. Polypeptide lead compounds, for example, can be obtained by screening antibacterial peptides from human, animal, bacterial, or bacteriophage origin.

The search for new classes of antibiotics either through identification or new targets of drug discovery programs is a logical approach (Fig. 38.6).

Until now all antibiotic classes with one or two exceptions are only directed at DNA replication, protein synthesis, and cell wall synthesis.

Traditionally, new antibiotics have been identified from biological sources, for example, actinomycetes and fungi from soil samples. The initial screening procedure is based on the effect of the new compound and on bacterial multiplication. This procedure is less time-consuming and expensive than the target-based approach. However, screening large collections of (biological) compounds for antimicrobial activity is a rather random procedure, depending on a "lucky hit" rather than on rational drug design. Moreover, no direct information is gained on the antibiotic target of the lead compound, which may hamper its further optimization. As all higher organisms produce peptides with antibacterial activity, numerous proteins and polypeptides with antibiotic activity have now been isolated and/or cloned from a broad range of both simple and complex organisms, including humans, animals, bacteria, and bacteriophages. Human agents with microbicidal and pro- or anti-inflammatory activities, for example, include β-defensins and cathelicidins. Defensins are broad-spectrum antimicrobial molecules that act against infectious agents and play an important role in the innate immune defense of vertebrates. These molecules exhibit a wide range of antimicrobial activities [4]. Another, very promising approach that regained interest is the use of bacteriophages. Phage host-cell lysis proteins, encoded by holins and amidases, have potent and very specific antibacterial activity, even though they do not enter the bacterial cell [12].

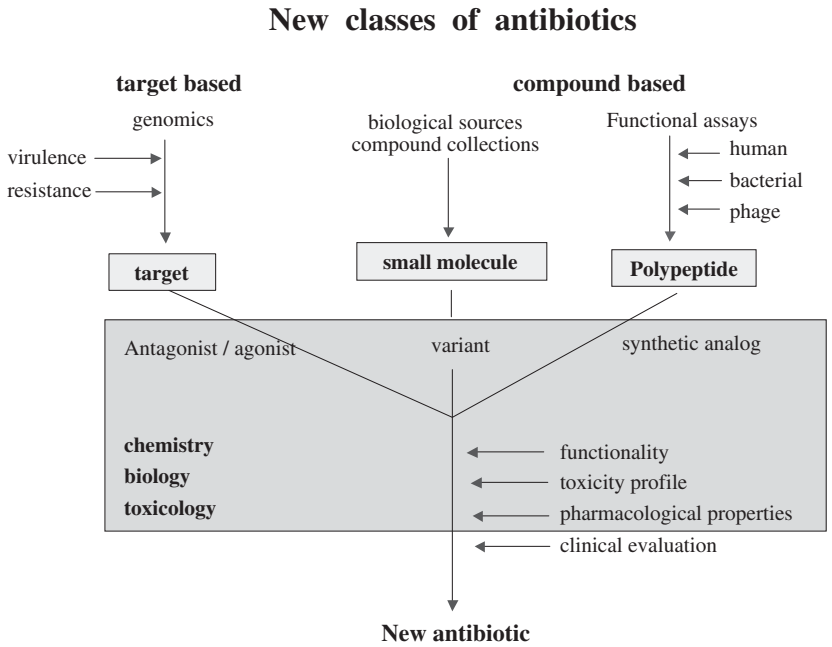

Fig. 38.6. Different approaches to identify new target and lead compounds for the development of new antibiotics.

A drawback of these compounds is that they are proteins, and there are several potential problems with proteins. First, they are large molecules. This means that normally they enter cells and therefore cannot attack targets within the (bacterial) cells. Secondly, their use may lead to an immune response, rendering further treatment useless. Thirdly, proteins are hard to modify to improve their pharmacodynamic properties. Nevertheless, these molecules may provide useful alternatives for antibiotics. The value of potentially therapeutic proteins would be greatly enhanced when their binding of active site can be mimicked by small molecules that can be easily modified. However, these technologies are still in their infancy.

Research in genomics is providing a range of potentially new targets for new antibiotics. In this type of research, genomes of bacterial pathogens are compared and analyzed in order to identify those genes and biosynthetic pathways that are conserved and common to a broad spectrum of pathogens, but are absent in the human genome [8]. Targets may comprise essential or accessory (e.g., virulence) genes. Essential genes are those genes whose loss of function coincides with loss of viability. This approach has identified a large number of potentially essential genes in a number of important pathogens. However, it proves difficult to find appropriate molecules that are able to penetrate the cell wall, interfere with the target and have no toxicity for humans.

Functional genomics can also be used to identify candidate targets. Analysis of the transcriptional response of a pathogen upon infection or induced by certain antibiotics may also herald new therapeutic targets (transcriptional genomics). These targets must be further validated by assessing their function or essentiality using molecular techniques such as transposon mutagenesis or antisense RNA. The interactions between lead compounds and biological targets can be explored and optimized using structural biology and traditional and combinatorial chemistry approaches.

Complementary Drugs: Complementary drugs interfere with intrinsic or acquired resistance mechanisms in order to extend the life and spectrum of important antibiotics. One important example includes the multidrug efflux pump inhibitors that are currently being developed.

Serious consideration should also be given to the possibilities of probiotics, which reduce the chance of colonization of the patient by resistant microorganisms, and to compounds that reduce the transfer of resistance determinants. Vaccination may also play a role in the reduction in the number of infection and thereby the need for antibiotic usage and consequent risk of resistance development. In this respect, however, drugs that can bolster the immune system during infections should not be excluded.

Vaccine Development: Vaccination is both an alternative and an addition to antibiotic therapy. In particular, newly developed conjugate vaccines have proven successful against infections with encapsulated bacteria. These vaccines include *Haemophilus influenzae, Sreptococcus pneumoniae*, and *Neisseria meningitidis*, but, in each case, only target the strains of some serotypes/groups. A new conjugate vaccine against *S. aureus* (Staph vax) is also showing some potential in clinical trials. Use of the conjugate pneumococcal vaccine has markedly decreased invasive pneumococcal disease in children less than 5 years of age. In the United States, this has resulted in a reduced prevalence of invasive diseases due to resistant strains because resistance is mainly found in strains of the serotypes covered by the vaccine. The conjugate vaccine, however, has little effect on the prevention of otitis media and may induce a replacement in the carriage of vaccine serotypes by nonvaccine serotypes [17]. Use of a conjugate pneumococcal vaccine in conjunction with educational intervention programs that promote appropriate and judicious use of antibiotic is a safe and effective means of decreasing the prevalence of invasive pneumococcal disease and drug resistance in the pediatric population and consequently decreasing the use of broad-spectrum antibiotic agents. In addition, vaccination against influenza may reduce secondary infections with *S. aureus* and *S. pneumoniae*, thereby further reducing the need for antibiotics.

38.12 PHARMACOGENOMICS

When a drug is given to a patient, the patient's response to the drug (e.g., antimicrobial agent) is very much individually determined. Pharmacokinetic parameters are often different from one patient to the other. Inter-individual variation is often due to genomic differences. Pharmacogenetics uses genetics to understand inter-individual variation in drug response. Pharmacogenomics uses a variety of technology approaches such as genetics, transcriptomics, proteomics, and metabonomics to understand the basis of inter-individual variation in clinical response to drugs.

"The transition from pharmacogenetics to pharmagenomics is important. Scientists are now taking a more holistic approach to understanding disease and drug response [9]. Since polymorphisms in DNA can lead to changes in RNA expression, altered protein expression or function, and changes to the metabolic profile of cells, additional disciplines (the so-called 'Omics' technologies comprising transcriptomics, proteomics and metabonomics) are now being embraced in addition to traditional genetics" (Fig. 38.7). The result of the clinical studies can help us to better prescribe antibiotics. Some patients are able to more rapidly metabolize drugs than others. These patients need to take higher dosages. The realization that pharmacogenomics can differentiate subpopulations of patients who are more likely to have a specific response to treatment may lead to improved therapy. This ability has broadened the application of pharmagenomics in the drug discovery pipeline. The application of these technologies early in the drug discovery process allows preclinical assessment of the impact of genetic variation and identifies potential issues up front that can be addressed in the clinical program.

So, pharmacogenomics will also be important in designing clinical trials. Thus, the application of pharmacogenetics in the drug discovery and development process is becoming

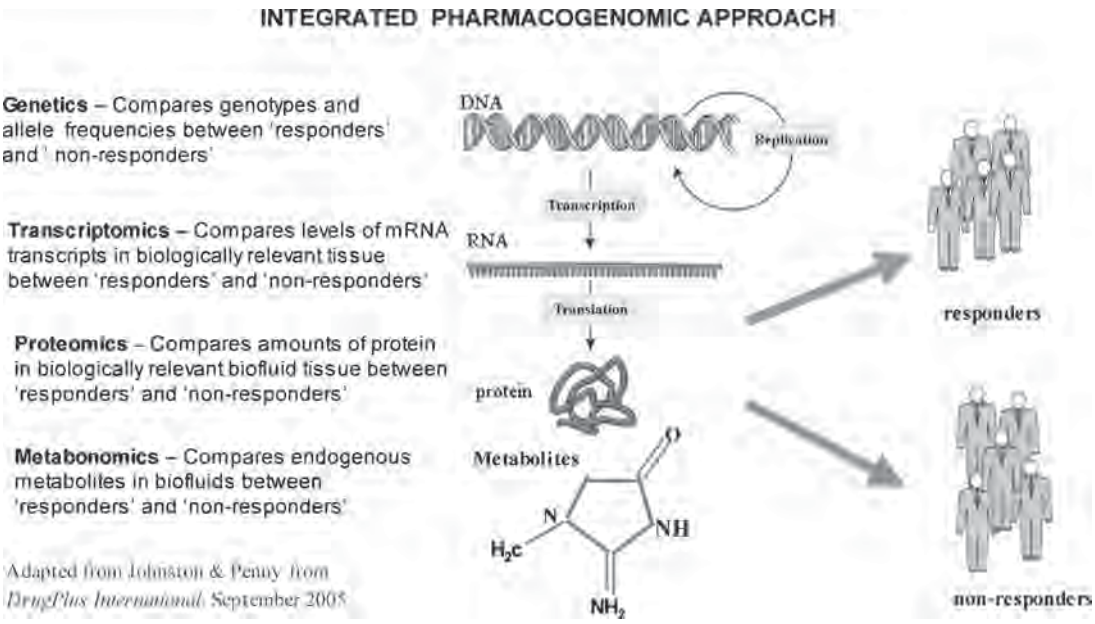

Fig. 38.7. Integrated pharmacogenomic approach to discern responder from nonresponders to drugs. (Adapted from Johnston G and Penny M, DrugPlus International 2005;4:19–22.) See color plates.

a very important tool to tailor new patients for appropriate patient-groups.

In addition, the range of other "omics" technologies are applied in a variety of ways to support preclinical drug discovery [8] (Fig. 38.8).

38.13 CONCLUSION

The revolution in biomedical research and information technology will be felt in the various Departments of Diagnostic Medicine. Eventually, all of the subdisciplines of Diagnostic Medicine will merge. Differences in techniques will disappear and differences between, for example, pathology and radiology will fade. Genome-based diagnostics will slowly integrate into microbiology, immunology, and even medicinal chemistry. As a result, clinicians/researchers will be able to make the correct diagnosis much faster and treat the patient with the most adequate therapy. Uniform treatment will become possible, and the quality of treatment will improve. In the end, treating patients will become cheaper: No irrelevant tests will be ordered, and the best cost/effectiveness will

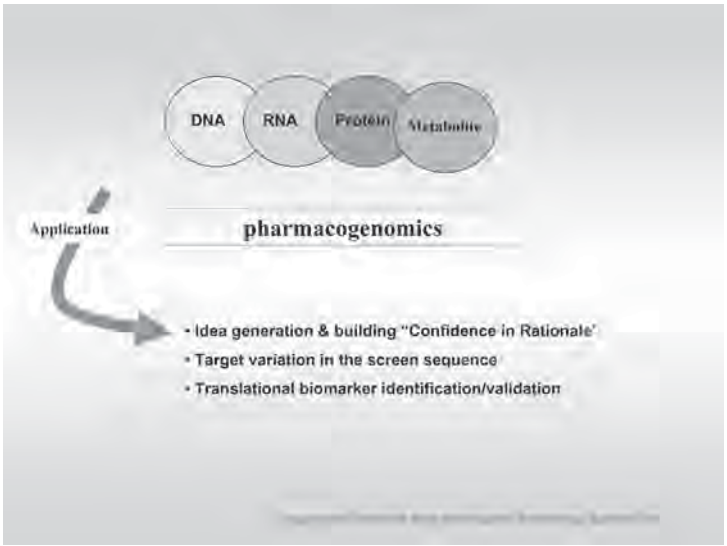

Fig. 38.8. Preclinical applications of pharmogenetics. (Adapted from Johnston G and Penny M, DrugPlus International 2005;04:19–22.)

be selected automatically. Waiting lists will disappear, and patient work-ups will be much faster. The training of doctors can be shortened; physicians will no longer need to have an encyclopedic memory as the computer will be his/her electronic memory.

The way we treat patients will change completely in the next two decades. For some doctors, the scenario of how medicine is evolving is full of horror. It appears as if the computer will take over the role of the doctor and the patient will become a number. In this scenario, however, the key role will not be played by the computer but by a compassionate physician. Only a compassionate doctor is capable of being the human factor and, in the electronic world, the patient will desperately need an empathic doctor. Working with a computer is not difficult; showing compassion, however, is a matter of training, education, and, above all, attitude.

The Internet revolution, that is, the rapid availability of electronic evidence-based information, will ultimately benefit the patient. The development of up-to-date expert systems and computer information technology will change the way microbiology is conducted. By integrating all of the diagnostic data of a patient, unnecessary tests will be avoided. As a result of the invasion of information technology into diagnostic medicine, new diagnostic methods will slowly evolve. Problems of contamination have to be solved and completely new diagnostic assays developed. Although the methods of Koch and Pasteur are currently still the cornerstones of the microbiology laboratory, results of proteomic and genomic research will undoubtedly change the face of microbiology. These changes will ultimately lead to the redesigning of processes in the microbiology laboratory. The resistance problem will also bring about new investments by (big) pharmaceutical companies in the area of infectious diseases. New ways of discovering antimicrobial agents will be used, with the ultimate goal to provide the physician with better treatment options.

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CHAPTER 39

Mobilizing the Scientific Community for the Patient's Benefit: At the Crossroads of Fundamental and Applied Science

K. Victoir

Today, one-third of the world's population lacks access to essential medicines; in the poorest parts of Africa and Asia this figure rises to one-half. Health care workers active in Southern countries have witnessed too many people in large parts of the world suffering from infectious diseases that cannot be treated because they are not properly diagnosed, the necessary medicines are too expensive, too toxic or not effective, or they are no longer produced. Large numbers of humanitarian organizations have been campaigning internationally to find long-term, sustainable solutions for this crisis. Initiatives such as the Médecins Sans Frontières (MSF) Campaign Access to Essential Medicines, FIND, Medicines for Malaria Venture (MMV), the Drugs for Neglected Disease Initiative (DNDi), Institute for One World Health (iOWH), OXFAM, and other organizations, are examples of organizations trying to overcome the barriers inhibiting access to proper diagnosis and treatment, especially in developing countries. These initiatives are striving toward lowering the prices of existing medicines, bringing abandoned drugs back into production, accelerating the creation of appropriate and affordable diagnostics, stimulating research and development for diseases that primarily affect the poor, and overcoming other barriers to health care access.

39.1 WHAT ARE NEGLECTED DISEASES?

Neglected diseases are those that mainly affect people in poor countries, although because a small market may exist in wealthy countries some R&D is ongoing [10], such as malaria and tuberculosis, for example. Although HIV/AIDS cannot be considered as a neglected disease, some aspects of disease control fall within the definition because of specific conditions in Southern countries and the North's neglect of these particular contexts [20].

Most neglected diseases exclusively affect people from developing countries who are too poor to pay for any kind of treatment or diagnosis. Because they do not constitute a lucrative market, they are of minor interest to private health-related industries. Research is therefore mainly restricted to public research institutes and the development of applied tools such as diagnostics and effective drugs are dramatically lacking. Examples of the most neglected diseases are leishmaniasis, trypanosomiasis (African and American), buruli ulcer, ...

Any medical team in the field is first and foremost preoccupied with patients' needs, and consequently, it is also their duty to denounce the unmet needs they encounter and to communicate them to policy makers, the scientific

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

community (public as well as private), and the general public. Current disease control is a complex issue in which the scientific community, international policy makers such as WHO, funding agencies, and regional authorities should play predominant roles.

Although nongovernmental organizations are tackling a wide variety of complex and multifaceted issues involved in the attempt to remediate this phenomenon of neglected disease, this article aims to focus on the gaps between fundamental science and needs-driven applied research, as well as the gaps and failures of existing diagnostic and lab tools.

39.1.1 Identification of Patient Needs and the Barriers to Overcome

Despite a profusion of basic research on infectious agents, particularly Southern countries continue to lack effective medicines and sufficient, efficient, and affordable lab tools. The rationale given for basic research on the causative agents of these diseases is often the identification of new drug targets or a possible diagnostic application, but much of the research remains in the abstract and is for the most part not developed into improved treatments or tools that can benefit patients. More than any others, it is the poor populations that are at great risk: 58% of the malaria cases occur in the poorest 20% of the world's population [3], and because they do not make up a lucrative commercial market, the high failure rate and the resulting high cost of drug development and proper diagnostics discourage scientists, funding agencies, and industry from investing time and money.

To achieve more successful needs-driven research, several gaps between different scientific fields must be bridged. Academic scientists and health care workers should be brought together more regularly to exchange knowledge and ideas on the needs encountered in the field and possible solutions. Communication among the different disciplines working on related diseases is currently inadequate, which contributes to widening the gap; fragmentation reduces the chances of sharing achievements across disciplines. Therefore, a meeting point between basic and applied research should be set up in such a way that joining forces to specifically address these needs would be perceived as more attractive. A common objective is needed, in particular the common motivation to contribute to addressing the medical needs of the world's patients.

Monitoring tools have become more and more important in supporting disease outbreak management and follow-up. Improved and more widely available tools would facilitate the control of many widespread infectious diseases in developing countries. *Diagnosis of neglected diseases is often a multistep process of screening, diagnostic confirmation, using one or more methods, followed by, in some cases such as human African trypanosomiasis (HAT), stage determination and finally monitoring of cure.*

For NGOs providing medical care to people who most need it and who are very often in difficult situations, diagnostic and screening tools should be field-friendly. The ideal tool for use in the field is *cheap, sensitive, specific, easy to use (3–4 steps), safe, fast (a few hours at most to result), and large-scale application should be feasible.* In some situations, it is not possible to implement such a rapid test because it simply does not yet exist. Indeed, despite scientific efforts leading to enhanced disease control in the North, the problem of the availability of effective medicines, of efficient and affordable tools is still dramatic, especially for Southern countries. For example, although HIV/AIDS is a highly monitored infectious disease in Northern countries, it is difficult for patients in Southern countries to have any follow-up for viral load and CD4+ count [15], because the existing technology is too expensive and too sophisticated to implement in rural areas, with the result that the follow up of HIV/AIDS is neglected in these areas. Viral load determination is very important because early determination of therapy failure can save lives. Without viral load monitoring, it can take months, even years, before clinical symptoms associated with therapy failure appear and consequently precious time to treat the patient with second-line medication will be lost.

For human African trypanosomiasis, the best available screening test is a serological test in use for more than 20 years [19]; although effective in a large part of the endemic area, very few initiatives are being supported to innovate, standardize, make the test available on a large scale, and include *all* clinically important and emerging strains. Diagnostic confirmation relies on the finding of trypanosomes in the blood, lymph nodes, or cerebrospinal fluid (CSF). Unfortunately, it is estimated that 20–30% of patients are missed by the standard parasitological techniques. Staging of the disease is a key step that allows classification of the patient into the first (hemolymphatic) stage or the second (meningoencephalitic) stage of the disease. In the absence of reliable blood tests able to detect central nervous system invasion of the parasite, HAT staging continues to rely on a CSF examination.

39.1.1.1 Monitoring resistance The rise and spread of drug resistance is more and more preoccupying because second-line medication is not always available and the resistance increases the cost and complexity of cure. Early-stage detection of an infection with resistant pathogens can save time, energy, and scarce financial resources in a patient's cure [18]. Although resistance is an intensively studied subject in the most important pathogens that are endemic in Southern countries [2,6,9,26,27], very few and in most cases no rapid, cheap tests are available to detect the presence of resistant infectious agents. The ideal test would directly evidence the presence of viable resistant pathogens in a patient's sample. With the presently available techniques, this means demonstrating specific antigens or nucleic acid sequences (preferably RNA). Here again the challenge to the scientific world will be to translate the knowledge acquired by genome and proteomic analyses into a user-friendly, cheap, and rapid field test.

39.1.1.2 Congenital transmission Neglected among the neglected diseases are the tools for monitoring congenital transmission. As many rapid tests are based on immunochro-

matography, which for the most part detects antibodies, these tests cannot be used in the first months after birth because of important cross-reactions with maternally transmitted antibodies. Although early detection and treatment can save lives, if adequate medication is available, mother-to-child transmission is not monitored and sometimes barely studied. Yet a recent study on *Trypanosoma cruzi* showed that using the right pathological analysis, relatively simple methods such as parasite detection using the microhematocrit concentration method on umbilical cord blood can be recommended for diagnosis [5]. Together with the development of pediatric drug doses, more efforts should be put into the development of easy tests for the monitoring and the early detection of transmitted pathogens in the foetus and the neonate.

39.1.1.3 Development of tools Molecular biology introduced the genomic and proteomic era and contributed a wealth of techniques and molecular targets. Indeed, qualitative and quantitative detection techniques have had a major impact on diagnostics. Both signal and target amplification-based systems are now used routinely in most laboratories in developed countries. Especially with improved and automated nucleic acid sample isolation techniques, as well as real-time detection methods [1,22,24], a new generation of assays for infectious agents is being developed: microarray detection [11,12]; nucleic acid sequence-based amplification (NASBA) technology — a sensitive, isothermal, transcription-based amplification system designed to detect RNA targets [8] – loop-mediated isothermal amplification (LAMP) [16]; and proteomic signature analysis [21].

In contrast to the classical parasitological and microscope analysis, these technological improvements make it possible to generate results with a very short turnaround time and with a very sensitive character.

However, molecular diagnostic tests require specific sophisticated equipment and electricity, which are not always available in field situations. Especially in poor endemic countries, laboratory facilities are very often nonexistant and the power supply very unstable.

Developing the best possible tools adapted to resource-poor settings is one of today's challenges demanding both scientific creativity and sufficient means. A first step would be determining which information such a test should give and to what extent northern-monitoring requirements should or should not be transferred to the Southern context. According to the requirements of the particular setting, new tools might not need to be as high-performance but rather a little more user- and wallet-friendly. Consequently, once the needs are established, the goal should not be to adapt existing sophisticated tools to field situations, but to develop the best possible tool.

Alternatively, if an adapted field tool is not yet available and if simplification of the technique is not yet possible, it may be possible to work at different levels of care depending on the existing technical, logistical, and skill possibilities of the health care system in place. In this system, it might not be necessary for all health care stations to function at the same specificity. For example, that very remote field stations could deal only with sample collection and detection by simple methods considered as "alarm tools," intermediate centers would do the actual diagnosis and biochemistry, and reference centers would monitor and treat complicated cases in addition to collecting samples, using alarm tools, and conducting diagnosis and biochemical analysis. The immediate need in such a system would be to organize a sample transport chain enabling a maximum of sample use at the different types of centers, with the additional challenge of applying specialized techniques on simple user-friendly and transportable start material (e.g., blood on filter paper).

It remains important that, in addition to using modern technology, monitoring professionals not lose the knowledge of the morphology and ecology of the pathogens they are dealing with. Indeed, although microscope and culture demand trained personnel and are very often time-consuming and require strict transport conditions, they are still the only means for diagnosis in remote areas (Fig. 39.1).

39.1.1.4 Standardization of diagnostic tools Molecular and serological diagnostic and detection methods require the use off standardized materials and participation in

BOX 39.1.

Polymerase chain reaction (PCR): Rapid amplification of a specific nucleic acid sequence by the use of different temperatures and an enzyme called Taq-polymerase.

Reverse transcriptase-polymerase chain reaction (RT-PCR): RNA amplification method whereby RNA is reverse transcribed into cDNA by an enzyme called reverse transcriptase and where the signal is amplified by an additional polymerase chain reaction.

Microarray detection: Detection of the expression of specific nucleic acid sequences. Signals can be obtained by hybridizing a fluorescently labeled nucleic probe, which has been prepared from cellular RNA, to DNA targets that have been immobilized on a solid support (spots on the microarray slide).

Nucleic acid sequence-based amplification (NASBA): Isothermal RNA amplification method, which can be performed without the use of specific equipment such as a thermocycler. Detection can be done by electrochemiluminescence, agarose gels.

Loop-mediated isothermal amplification (LAMP): Amplification method of DNA under isothermal conditions. The amplification, and thus the result, can be monitored spectrophotometrically or with the naked eye without the use of specialized detection methods.

Proteomic signature analysis: Analysis of the specific features of a protein by, for example, spectrophotometrical or mass spectrometrical analysis.

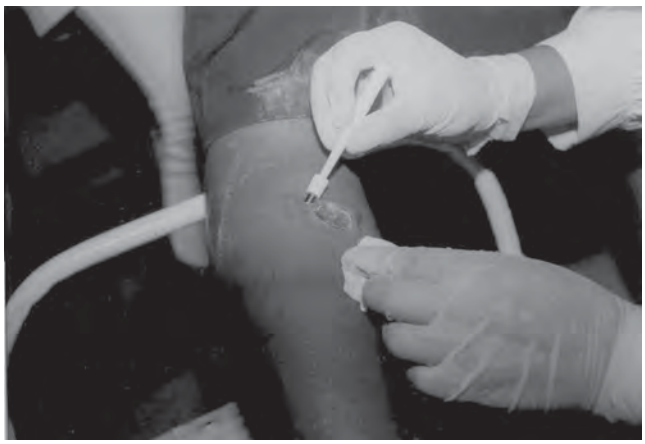

Fig. 39.1. Taking a biopsy of a lesion caused by *Leishmania ssp*.

international quality control programs. Unlike the very strict legislation and tests concerning the development and use of new drugs, there is no standardization for the legislation and use of diagnostic tools in terms of methods used, control of specificity, quality and quantity. Diagnosis of American trypanosomiasis, for example, is based on the presence of antibodies against the protozoan parasite *T. cruzi* in the serum of infected individuals. These antibodies are usually detected by an array of serological tests. If these tests are performed by trained technicians, with good-quality kits, it is possible to define the status of more than 98% of serum samples [7]. Nevertheless, in busy, routine diagnostic laboratories, hospitals, blood banks, and among others, the use of commercially available kits might yield much less than this 98%. Variation in reproducibility and reliability of these tests has been reported and explained by poor standardization of the reagents [4].

Although the European Community requested a reference measurement system (RMS) to be set up for each and every quantity measured or determined in laboratory medicine, many gaps remain in the standardization of lab monitoring tools [17].

Due to inadequate convention and regulation, there has been a proliferation of in-house diagnostic methods: some could be very valuable for the international community and others may add no additional value to existing tests or needs, comparable to the "me-too" drugs. As a result, preliminary analysis is sometimes necessary, for example, comparing different PCR primes, antibodies, and so on, before a corresponding diagnostic survey can be started. There is little uniformity in the checklist of organisms and their quantity and genetic variability in testing possible cross-reactions. In commercially available tests, it is rarely mentioned if any cross-reactivity has been observed, and to what extent this has been checked. Even if no cross-reaction is detected with one reference strain that has been in culture for many years and might no longer be representative of the actual field situation, cross-reactivity with that organism or species cannot systematically be ruled out. Additionally, tests designed for diseases that are also endemic in the North too often neglect to take endemic strains of the South into consideration; such has been the case for certain African HIV strains in the past. Therefore, detection techniques should cover a broad genetic diversity as possible, especially because of the ability to characterize pathogens more easily by sequencing opens new possibilities for epidemiological studies.

An accurate picture of which relevant strains and species are circulating in the field and therefore the exact discriminatory power of a diagnostic test requires an overview of the epidemiological situation of the area of interest. Close collaboration with (molecular) epidemiologists is therefore recommended. This could mean a fixed panel of organisms to be tested for each infectious agent that is the subject of a diagnostic evaluation. This panel would be freely available as a sero-library or cryo-bank to any group willing to evaluate the value of a developed test. This would make it much easier to compare the discriminatory power of various existing or diagnostic tests under evaluation.

Finally, introducing universal internal monitoring of the entire procedure would guarantee the accuracy of the results generated.

To bring about applied research that serves the interests of the public, the existing needs must be identified and the subject of applied disciplines (diagnostics, epidemiology, drug development, etc.) reevaluated.

Achieving this goal will require

- a continued effort to identify, characterize, and widely publicize neglected medical needs and accordingly develop and promote a *needs-based R&D agenda*;
- new sources of *funding* or a restructuring of old sources to the benefit of neglected diseases;
- promote a *multidisciplinary approach* to address the needs-based health R&D agenda.

39.1.2 Developing and Promoting a Needs-Based R&D Agenda

Those diseases and medical conditions throughout the world that continue to lack sufficient pharmaceuticals and monitoring tools should be identified, relevant questions should be carefully listed (what and why), and the context in which they will be used described before possible R&D solutions are sought (e.g., for HIV/AIDS, the range of viral loads that should be covered, for parasitological infections determination of the extent to which molecular strain or species identification is necessary for adequate treatment, etc.). This requires a sustained dialogue between patients, doctors, and health care workers close to patients on the one hand and the research community with the tools and knowledge to address these needs on the other.

Based on this interaction, a concrete *knowledge- and needs-based R&D agenda* with short-, medium-, and long-term goals for new or improved health care tools can be developed for each of the neglected diseases or conditions. This agenda should assist policy makers, funding agencies, and the research

community in setting the right priorities to address the needs of developing countries effectively. The next critical step is for governments and international organizations to carefully examine how they can contribute to opening up the bottlenecks that currently restrict development of new treatments and monitoring tools.

39.1.3 Funding

39.1.3.1 Financing Although the prospect of a limited market restricts the investment of the private sector in the search for the necessary tools to combat neglected diseases, governments are responsible for ensuring that the progress of science and technology also benefits the less wealthy. The public research community has a vital role to play in addressing the otherwise neglected health needs. Therefore, a needs-driven R&D agenda where monitoring tools and their use are reconsidered and put high on the agenda of responsible funding agencies should be implemented, so that available funds for disease control are redirected to the benefit of affordable, efficient, easy-to-use diagnostics, monitoring tools, or transport chains of samples to reference centers.

Applied research for neglected diseases will be perceived as attractive only if it is considered challenging and supported by the necessary funding. No money, no exciting science, and no retention of experienced personnel. Unfortunately, the funding sources for applied science or field research on neglected diseases are very limited in the public sector. It is only very recently that funds have become available, but this cannot be considered sufficient, until, in addition to limited WHO and EC financing, temporary funds from private organizations such as the Gates Foundation are willing to invest in monitoring tools for the South. Much more durable, long-term solutions should be advocated for and implemented. Furthermore, civil society should be sensitized to neglected diseases to encourage individuals to donate funds.

Initiatives such as MSF's Campaign for Access to Essential Medicines, DNDi, MMV, OXFAM, iOWH and others continue to successfully mobilize the scientific and political communities.

39.1.3.2 Evaluation of scientific work on neglected diseases The main criteria used today to evaluate scientific research, which in turn guides promotion, funding and reputation, are the Science Citation Index (SCI) and the journal impact factors (JIF). As discussed in Schoonbaert et al. [25], these criteria are not without problems, especially in fields such as neglected diseases and applied sciences. High-ranking journals almost exclusively publish major advances in fundamental knowledge, very often generated by sophisticated molecular and immunological research. Applied research on tropical diseases, field work, screening for active compounds, evaluation of diagnostic tools, and so on, on the other hand, is not considered as innovative, insightful, and pioneering and is consequently relegated to journals with a more modest reach. This is unfortunate, as the bottom line is that where you are published matters more than what you publish, especially in the eyes of funding agencies.

It is important to stimulate the debate on the relevance of the current rating system for applied research in neglected diseases and propose more appropriate evaluation criteria such as the potential impact of the work, the applicability of what is proposed, ... In particular, research publishers, funding agencies and policy makers (Ministries of Health and Education, etc.) that award grants and manage research programs need to be sensitized to this situation and asked to judge the quality of the work published and not merely the number of citations in journals.

39.1.4 A Multidisciplinary Approach

39.1.4.1 Stimulation of a multidisciplinary approach To address and resolve a field-related problem pertaining to a neglected disease indeed requires the collaboration of health care workers, biologists, biochemists, molecular biologists, physicians, epidemiologists, veterinarians, pharmacists, and others. It is crucial to involve health care workers from endemic areas who have direct experience with neglected diseases and can provide a broader view on the reality of disease control, such as advice on the administration of drugs or disease eradication strategies in politically and economically unstable situations.

A multidisciplinary approach would mean first that common goals are identified and second that each expert would have a specific role to play. Interactive research strategies with common goals must be initiated, reflecting the global health care R&D agenda.

Although monitoring tools should be very specific, a horizontal approach across different disciplines (e.g., with epidemiologists and veterinarians) bridging the gap between different organisms should be advocated. As some infectious agents are also endemic in Northern countries and given their pandemic nature, such as HIV/AIDS, more funding possibilities have been available and a greater variety of monitoring tools have been developed. Some of these techniques are also applicable for the detection and quantification of other pathogens than the ones they were originally designed for (e.g., NASBA [24], LAMP [16], blood stabilizing agents [14]).

Coinfections should be increasingly taken into account when designing a novel monitoring tool. Indeed, a co-infected patient with a protozoan parasite and HIV/AIDS or with a mycobacterium and HIV/AIDS might have a very altered clinical pattern. Moreover, because of immunosuppression serological tests might have an unreliable outcome. As the HIV/AIDS epidemic is continuing to expand, especially in Southern countries, the patterns generated by coinfections should be taken into consideration when designing and evaluating a novel diagnostic or monitoring tool.

39.1.4.2. Knowledge sharing and interaction As explained above, international and multidisciplinary communication concerning monitoring tools and coinfections is

very important. Indeed, part of the gap between fundamental science and applied and field research is the dissociation of research and disease control. We need to bring health care personnel working directly with patients in contact with researchers working in the laboratory. Communication should flow in both directions, not only where research will be guided by the needs exposed by those working toward disease control but also in the opposite direction so that people working in the field come to an understanding of what can be used of the diverse discoveries made by those doing research. Nongovernmental organizations could play an important role in creating this horizontal communication because they are in a good position to stimulate discussions between the different parties (scientists, health care personnel, medical staff, policy makers, etc.) involved in monitoring various infectious agents (Fig. 39.2).

39.1.4.3 Global access to knowledge Open communication is seen as an attractive proposition by most scientists, as a means to ensure that researchers in the South receive news of the latest developments *via* not only traditional, subscription-based scientific journals, inaccessible to most workers in developing countries, but also free online or local journals and a centralized, accessible resource for the different disciplines interested in neglected diseases.

To make multidisciplinary communication and results globally accessible, especially for developing countries, a coordinated, open-access information system is needed, for example, an Internet site with links to different initiatives already in existence. The objective would be to create a well-organized central forum on the Internet.

An important catalyst to open-access communication was given by the Open Society Institute (OSI), which promotes open societies by shaping government policy and supporting education, media, and public health reforms. They supported open-access projects such as the Directory of Open Access Journals (DOAJ) and grants to support the publication of articles by authors from developing countries in open-access journals. Two leading projects in open-access publishing are the Public Library of Science and BioMed Central. PloS is a nonprofit organization made up of scientists and physicians committed to making the world's scientific and medical literature a public resource. In October 2003, PloS started free online access to all its journals, such as *PloS Biology*, and plans to launch another dozen titles by 2008. BioMed Central is an independent publishing company established in the UK and publishes over 100 peer-reviewed open-access journals covering all areas or biology and medicine. Citations of open-access articles are rising and in the current rating system does not lower the impact factor of the journal [13].

Fig. 39.2. Laboratory research: disconnected!

In the field of parasitology, a number of interesting sites have been launched, for example, ILN (International *Leishmania* Network), sites offering free access to certain journals for researchers in the South, free *Kinetoplastidae* journals, an information site for medicine and tropical health for Africa, and sites for publications from developing countries not listed in *PubMed (ExtraMed)*. Sites that compile and promote national publications that are locally produced (e.g., BERIME [http://www.berime.br] on parasitology, toxicology, biochemistry, etc., in Latin America) should be encouraged. A centralized neglected diseases site where all this currently fragmented information is available, as well as useful links to sites already in existence, eventually not restricted only to publications but also making available free tools (e.g., software, see also Section 39.1.4.5) could be of great value. Particularly in countries where, if computers and support are available, searches are regularly interrupted by power failures making a browse on the Internet a real adventure. Fortunately, the open-access movement is gaining support and will undoubtedly benefit researchers from the South.

39.1.4.4 From the academic lab to production: training and retention of experienced personnel In drug development, the barriers between academic discovery and small-scale use to large-scale production are many. Indeed, most scientists are not familiar with the steps involved in marketing and large-scale production. Traditionally, pharmaceutical companies train the medical chemists they employ for drug development, and medicinal chemistry expertise is not yet very well developed in the public sector. The same holds true for other specific areas in diagnostics development, such as laboratory evaluation, toxicology, or regulatory affairs.

Specialized fellowships could be initiated in collaboration with pharmaceutical industries and biotech companies. Creating a novel discipline or network to reduce the gap and encourage the development of the needed tools has been suggested. New knowledge acquired by fundamental scientists would be applied to develop tools for controlling neglected diseases. Training could lead to masters or doctoral degrees, for example, which would cover fields from biology to regulatory affairs with stops in toxicity studies, efficacy studies, pilot production, and clinical trials (an original idea proposed by Dr. F. Modabber). Scientists from private companies and academics should be involved in this scheme, bringing both research fields into closer contact and creating the possibility for specialized fellowships. Such initiatives attempting to

bridge the training and research gaps should be supported and made accessible to a wide audience in the South.

Creation of postdoctoral positions and positions for other scientific personnel providing the opportunity to gain experience in the field should be encouraged. An initiative aiming to bring scientists into the field is Scientists Without Borders completely equipped containers will be put in endemic countries and will serve as field-laboratories. Basic scientists will experience firsthand the problems inherent to field work and hopefully (re)orient their fundamental research toward a needs-driven framework. Organizers hope to create more training opportunities for local scientists by involving them in the project.

Ph.D. and postdoctoral students carry out most of the research activities in the academic world, even more so in the field of neglected diseases where collaborations with industry are more difficult to achieve. Once these students are experienced researchers, the limited career perspectives in the public research community, the lack of funding opportunities, and the limited overall appreciation of an applied or field type of activity make them move away from the neglected diseases to financially secure and more attractive sectors. To stop this intersectoral migration and also to motivate enthusiastic and experienced scientists, these individuals must be given sufficient remuneration to allow them to continue in the field of noncommercial drug and tool development for field applications. Therefore, criteria used by the funding agencies and institutions involved should be modified regarding applied research for neglected diseases. Recruitment and retention of experienced people plays a key role in maintaining a continuity of capability and expertise.

In the long run, multidisciplinary courses for Ph.D. and postdoctoral students could be instituted, allowing greater information exchange between students and senior scientists. This exchange concerning needs-driven research (problems, necessities, challenges, successes) should be stimulated at different stages of a scientific career starting at the student level, as these are tomorrow's researchers, as well as field workers and health care providers. It is important, therefore, that awareness begin in schools and universities. Many young scientists, in this postgenomic age, understand how to identify candidate genes for therapy, for example, but are unable to apply this understanding to the overall biological system. Multidisciplinary approaches will help them see the forest as well as the trees.

39.1.4.5 Continued access to appropriate tools

Another weak link in the development chain is technical continuity. Although the private sector is making some effort toward neglected diseases, many useful compounds and technical skills remain locked up by patents or commercial barriers. We believe, however, that the collaboration between academic and industrial research should be encouraged (start-up companies, pharmaceutical industries, biotech industries). Initiatives created to tackle similar issues could be studied and lessons learned from them.

Such an initiative is The Biological Innovation for Open Society (BIOS), which argues that research tools should be freely available: operating systems, programming languages, and standards are shared by the open-source software community (http://www.bios.net). There is indeed a mounting concern that poorer nations are being further disenfranchised by richer countries owning and controlling enabling technologies. BIOS is aiming to help on several fronts. Its intellectual-property database and associated informatics promise to bring more transparency to the opaque patent web and provide tools to guide decision making when choosing technologies. The aim is also to provide an Internet-based mechanism to bring networks of researchers together to cooperate on specific technology development projects.

This example emphasizes the importance of an inventory or database of interested players (North and South, tropical medicine institutes, pharmaceutical industries, biotech companies, international organizations, etc.), who have both the capability and capacity to assist in a particular phase of the development process. Once the gaps in the process are identified, the appropriate players can be used to fill those gaps.

An additional barrier for laboratories in the South is the access to appropriate and affordable equipment, materials and reagents. A survey in *Nature* revealed that people in poorer countries sometimes pay up to 70% more than their colleagues in wealthier countries [23]. Local ministries of economy and concerned companies should be sensitized to the problem and solutions should be sought.

39.2 CONCLUSION

The social vulnerability of people living in poor countries requires a clearer understanding of the context to improve the deployment, access, quality and use of current scientific knowledge and inventiveness and move it in the direction of developing field-friendly monitoring tools.

Effective actions for disease control demand rational public policies, health education, appropriate market forces and coordination between the different research players. More networks and better communication between different research disciplines, institutions and health care agencies should be established. The challenge is to translate advanced technical abilities into the low-cost, rapid diagnostic tests needed to improve the accuracy of diagnosis and accelerate the start of appropriate treatment in low-income endemic countries. Initiatives, institutions, and laboratories that are working in this direction should be encouraged and put into the spotlight.

There is definitely a positive momentum among scientists, the private sector, funding agencies, and so on, in support of needs-driven research on neglected diseases. There is a strong resolve to collaborate and communicate. This new drive should be encouraged, its sustainability ensured and transformed into concrete action.

BOX 39.2 INTERNET SITES AND EMAIL LINKS

Campaign for Access to Essential Medicines: http://www.accessmed-msf.org/
Medicines for Malaria Venture (MMV): http://www.mmv.org/
OXFAM: http://www.oxfam.org.uk/
Institute for One World's Health: http://www.oneworldhealth.org/
Drugs for Neglected Diseases initiative: http://www.dndi.org/
FIND diagnostics: http://www.finddiagnostics.org/
Public Library of Science: PloS, http://www.plos.org/
BioMed Central: http://www.biomedcentral.com/
Scientists Without Borders: csf@sb-roscoff.fr

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CHAPTER 40

Infectious Diseases and Arts

Pierre Vidal,[1] Myrtille Tibayrenc,[2] and Jean-Paul Gonzalez[3]

[1]*Anthropologist, French Ministry of Foreign Affairs, Pays de Sault, Aude, France*

[2]*Allaince française, Bangkok, Thailand*

[3]*Institut de Recherche pour le Développement, IRD, U178, Paris France & Mahidol University, Bangkok, Thailand*

"When leaving his surgery on the morning of April Dr. Bernard Rieux felt something soft under his foot. It was a dead rat lying in the middle of the landing."

—*Albert Camus [12]*

FOREWORD

Treating infectious diseases as the theme of artistic production is attempting a truly impossible task that artists have nevertheless committed to: Art is many-faceted and diverse and references to infectious diseases abound.

In a highly exhaustive sense, any human creation, whether it be material or spiritual, stems from an art, which, however, requires certain aptitude, theoretical or technical knowledge resulting from learning. Often in the introduction, the art historian has claimed that there was no art, but that there were only artists. In other words, through an object or a work of art, individuals express their esthetic feeling—within a religious, social, or cultural context—a work of art sets off an emotion in the person who sees it or hears it or simply perceives it with his or her senses.

How many categories can artistic production be divided into? Let us say that it involves all the modes of expression of beauty and imagination. One art theoretician wrote "An object is a work of art in and of itself only in relation to an interpretation" [15]. Reference works suggest a variety of domains in which art is expressed: the dramatic arts, opera, the fine arts—still referred to as the spatial arts—including architecture, engraving, painting, sculpture, and, for more than 150 years now, photography. We can add to these the temporal arts (music, dance, and cinema, which has become the seventh art). There are the utilitarian arts (high-technology products), and, more contemporary still, television, or the eighth art, and comics, a ninth art. Reference can also be made more classically to applied arts, the decorative arts, performing arts, and, more specifically, to Art Nouveau, popular arts, the "poorman's art" (made of objects from daily life) [84].

With this profusion of domains, schools, and interpretations, we probed where our quest, our subject appeared unmistakably in image and text. Indeed, the fine arts, literature, and cinema provide us with a number of exemplary successes where our topic is expressed superbly; other types of artistic work, however, have also captured our imagination for their original interpretation of infectious diseases. Of course, we could have cited the marks of infectious diseases detected in the most recent rock paintings or in the artwork of ancient Egypt; and, at the frontier of history and medical art, we could have consulted the wonderful illustrated works of the sixteenth century used by the practitioners of medicine, who were then at the threshold of the passage from art to science. Finally, cinema—scientific in nature—today proposes beautiful examples of art at the service of medicine with rich and wonderfully human works on themes that have never been explored by the artist, such as onchocercosis with *Mara, the Lion's Eye* [93]: there are many others.

This text is also an essay; whether in a scattered or targeted manner, it is simply a pioneering look turned to artistic

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

representations of infectious medicine or the great pestilential calamities. We will take the liberty of a few particular critical comments on one work of art or another, where an infectious disease plays a role or helps to give particular light to the suffering of humanity.

The works of the fine arts—the collection of roughly 20 literary works that we will refer to, and the nearly 100 films that we will cite or comment on—seem to us to provide sufficiently rich light to the entire panoply of these productions, which we hope will incite the reader to further research.

We note that in artistic production, infectious diseases in their epidemic or endemic forms seem to have frightened the artists, some representing their own suffering in disease, conveying visual or auditory expressions to release the audience's emotion. But the relation of the arts to medicine, including what touches infectious diseases, is vast, from the person who suffers to the mystery of an elusive and insidious germ, from the microcosm to the macrocosm, from plants that heal to the imaginary.

Our purpose is threefold, each part concentrating on one domain: The fine arts, essentially drawing and painting, literature, in particular the novel, and cinema, excluding television production that today remains little or poorly referenced, or with little in the way of originality in its inquiry. Infectious diseases and epidemics find frequent representation in the narrative arts: Literature has the very early examples of the ancient texts and rarer illustrations in poetry; cinema begins in the 1930s, with a few even earlier examples from the days of silent films.

Art—it must be said again and again—expresses and transforms emotions and feelings; and the representation of death is present in all the arts. Even medical illustration, which is meant to be descriptive and informative, outside of emotion and feeling, often achieved the status of art in past centuries before the existence of photography. We have also taken a few examples in music and dance, often accompanied by a booklet referring to a literary work: For example, *La Dame aux Camélias* and its tearful (tragic, romantic) ending with the heroine's death from consumption; she would inspire a number of artists [5]. In addition, the extremely rapid progress of today's imagery techniques at times draw near artistic creation.

After the great themes of love, war, human life, and death, medicine, and especially disease, is an inspiration for artistic expression, such as tuberculosis during the romantic period and the diverse plagues that fed all fears.

Although illness, as the object of inspiration, did not greatly tempt the artist, it became imperative to the artist's creation by the drama it generated in society (e.g., syphilis), and when the artist himself suffered in his body and soul (e.g., through a handicap of traumatic origin as for Henri de Toulouse-Lautrec or even Frieda Khalo, or a chronic disease such as Vincent Van Gogh's epilepsy or his lead poisoning from the use of toxic pigments brought about by his passion for colors). In 1880, Pierre Auguste Renoir broke his right arm and painted with his left hand: He continued to paint until his death handicapped by repeated bouts with chronic arthritis. Paul Gauguin, afflicted with a heart condition and syphilis, never stopped producing. Infectious diseases are even more rarely treated, but some of them so profoundly marked the suffering populations as victims that they became the subject of artistic expression (e.g., the plague and its many representations in the dances of death).

For centuries, art and science—and philosophy—were confounded, and medical art referred to knowledge acquired by learning, a gift from he who exercised it. This confusion between science and art endured until the middle of the nineteenth century, at least in the West. Pasteur and Koch, among others, were the founders of a true medical science that the technology and progress of biology refined incessantly. As such, at the end of the twentieth century, doctors who believed they had finished with infectious diseases—and artists with their morbidity—found themselves plunged in yet another confrontation, which they did not expect: the emergence of new diseases, unheard of syndromes, new germs. These emerging diseases have imposed themselves on medical thinking and have awoken the ancestral fears of the great plagues that we believed forever buried.

Our conclusion will attempt to draw a few lessons from this endeavor: The meaning of infectious events in the collective unconscious and how art has used them to sensitize its audience. Beyond this use by the world of art, we propose a reflection on the use that is made of the infectious phenomenon in communications and the media, in today's economic stakes, in politics, and in beliefs.

40.1 THE FINE ARTS: PICTORIAL REPRESENTATIONS

Art is the essence, preserved for eternity, of the history of the human soul. Through the violence wreaked by the great epidemics of the past—and now those of the present—on the psyche of our populations, it is not surprising to find quite a rich pictorial representation of this theme in the history of art: works inspired by illness, religious images, historical paintings, medical painting, self-portraits of diseased artists, memorial plaques, and photographic documentaries.

Major catastrophes caused by the great infectious diseases resounded with particular force in each era, pushing artists to elude traditional artistic values in favor of an art closer and closer to reality, in perfect harmony with the intimate suffering of the human being.

Images intended to terrify, lure, mark the day's events, provide knowledge, exorcise evil, rebel, or immortalize: Representations of infectious diseases were organically transformed over the centuries, echoing human history. From the Middle Ages to the present, despite the extreme changes in the status of artistic works and of the artist, the myriad images inspired by epidemics are echoed in society in political, commercial, and even religious terms. In our contemporary epoch, AIDS is emblematic of this with its procession of false certainties based on ignorance as much as discrimination and stigmatization of the ill or social, ethnic, or geographical

group, access to health care a function of social injustice, the business stakes of medicines, the race to patents of every sort, the repeated failure of uncompleted vaccines, the punishment of the gods of believers.

> The creation of the diseased artist, produces without curing but helps live and die. The body plunged into dis-ease becomes the location and the enterprise whose inhabitants are not life and death but death in life and life in death. A purgatory that purges nothing, disease dispossesses us of our illusions of control. Man must find an ethical and esthetic front [14].

This chapter will look only into a few selected artistic works that we believed to be an interesting introduction to a subject that deserves more detailed research as well as the publication of an entire book.

40.1.1 The Roots of Art and Infectious Disease

Mirko Grmek and Danielle Gourevitch [42] wrote undoubtedly the first exhaustive work in the history of medicine treating "disease in ancient art," covering the period from the sixth century B.C. to the fourth century A.D. This is a vital era in the history of humanity, with most of the arts expressing a relation with illness, such as monumental sculpture, la coroplasty (casting of figurines or small statues in ancient Greece), fresco painting, vase painting, glyptics (the art of engraving on precious stones), numismatics, engraving on mirrors, mosaic, and so on. Although the period is important and the artistic representation rich, infectious disease in the strict sense appears only rarely and the greatest part of medical art is expressed in the representation of trauma (war scenes, care for the wounded) and diseases that were either chronic (obesity) or degenerative (goitre, dwarfism). In iconographic diagnostics can be found several skin lesions such as those resulting from smallpox, and, in sculpture, leprous leonine facies (a Hellenistic head in limestone, p. 248)—all of course based on a retrospective diagnosis whose symptoms shown can be confused with other infectious diseases that deform the face such as "lupus, nasopharyngeal cancer, actinomycosis, and so on" as well as the deformations of bony tuberculosis (Pott's disease, pp. 217–218). The divine arrows already symbolize the plague (Apollo provokes the plague by his arrows in the Greek army, which lays siege to Troy: Roman bas-relief—Capitole Museum, Rome, 1831 drawing by Inghirami, p. 96).

40.1.2 The Plague: Art, Terror, and Religion

The plague is one of the evils of humanity that incited the most debate. The first epidemic, Justinian's plague, named after the Byzantine emperor Justinian, raged throughout the Mediterranean basin from the sixth to the eight centuries of our era. It was first seen as a manifestation of divine vengeance or anger: God, no longer tolerant of the sins of man, had to exhort his clemency. The people of the time, attempting in vain to discover the origin of this devastating calamity, soon designated scapegoats. The Jews and the lepers were accused of spreading the evil by poisoning well water. Some resorted to pagan explanations: Eclipses of the moon or the sun, shooting stars, and comets were also blamed. The Middle Ages is a key time in history in terms of popular beliefs and new religions (considered as heretical by the Catholic Church). This is why the pictorial art relating to this scourge is abundant and diverse. We also see appear at this time, with the new techniques of image reproduction such as engraving, illustrated medical treatises, plates of medicinal plants, and diverse medical instruments involving the plague. We find illustrations of animals and the lack of hygiene illustrated as responsible agents. The health notice of 1628 drawn up by Lyon doctors claimed that "the embryo and seminary of the pestilence stems from the impurity and dirtiness of the body" [69]. As soon as it appeared in the West, the plague was associated with religious iconography. Christian art of the Middle Ages inspired by Roman art gave each representation a very strong symbolic impact, and thus the representations of the plague, death, and divine anger took on multiple forms: It was often represented by arrows (God throwing the arrows of the plague) see Fig. 40.1 and Saint Sebastian, who is, with Saint Roch, a protector saint for plague victims, was represented pierced with arrows (Saint Roch is marked with a plague bubo on his left thigh). These great epidemics, against which men were powerless, inspired a large body of iconographic works on death in its allegoric form or in a personified form: This marks the appearance of the themes of the death dance, the triumph of death, or the young girl and death, widely treated up to the Renaissance.

The goal of art is no longer to represent reality, as in Paleochristian art, but to place the faithful in direct contact

Fig. 40.1. Christ Throwing the Arrows of the Plague (fifteenth century) from the heavens Christ sends the arrows of the plague that that strike the body precisely where the buboes appear. Image from the fifteenth century where God can be seen sending the plague to man as arrows (Old School of Medicine, Iconographic Coll. Paris).

with a heavenly, spiritual world. Fantasy soon misleads the spirit, terrified by the morbid spectacle of the devastating illness. The works produced on this theme, at this time, take on the primary role of exorcising evil. They will be offerings to God and to the protector saints of the plague, to push away the disastrous consequences.

> Men applied all their artistic talent to exorcise evil, building chapels dedicated to the protector saints, so-called columns of "the plague" such as the one in Vienna, crucifixes . . . whereas diverse paintings, sculptures, and commemoratives helped ward off this misfortune [69].

The most famous of the masters illustrated it, such as Raphaël with *The Plague of Phrygia*, Rubens with *The Miracles of Saint Francis of Paola*, Van Dyck, *Saint Rosalie Interceding for the Plague-Stricken*, Nicolas Poussin, *The Plague of the Philistines*, Pierre Mignard, *The Plague in Epira*, Goya, *The Plague House*, Antoine Gros, *Napoleon visiting the pesthouse of Jaffa*, David, *Saint Roch Interceding for the Victims of the Plague*, Géricault, *The Plague Victims*, Gustave Moreau, *Saint Sebastian*, and so on [69].

40.1.2.1 The black death and the dance of death In the late Middle Ages, the black death already found expression in folk art (e.g., Saint Roch with a bubo on his thigh, Saint Sebastian and his arrow wounds, the dance of death, the carnivals). This is where the dance of death takes place: The plague strikes indiscriminately, it is the sign of death and it takes the rich and the poor alike in a deathly dance. The first artistic representation came from a fresco painted in the Innocents cemetery in Paris around 1424, which today no longer exists, but it remains well known for its ancient writings by Guyot Marchand, publisher and poet who saved the transcript before the its destruction in 1554. Then came the frescos of London (around 1430), Basel (a first one around 1440 and a second one around 1480), La Chaise-Dieu (approximately 1460–1470), and Lübeck (1463). During the second half of the fifteenth century, the death dances enjoyed ever-growing popularity.

The theme is also found in writing (see Chapter B) and in music (see Chapter D). Death was indeed often represented as a musical instrument. Premise of Dark Romanticism, this characteristic belongs to the rich repertory of the symbolism of death and appeared from the very first dances of death. The instrument calls to mind the seductive, attractive, slightly diabolic side of music's power of enchantment. Think of the song of the sirens, of Hamelin's flute player, and so on. Like them, Death charms man with its music.

Another consequence of the high death rate resulting from the plague epidemics was the birth of the theme of death's triumph. As Germany and France preferred the dance of death, Italy's tastes leaned toward this new genre. The egalitarian spirit remained, as in the dance of death and the legend of the Three Dead and Three Living: Death takes possession of both rich and poor, but this time, Death is not represented as an ordered dance, but rather in a furious combat with the living. The outcome of the battle is unequivocal: Man will be conquered by Death: it is unavoidable.

Fig. 40.2. *The Triumph of Death* by Pieter Bruegel the Elder (toward 1562). Death is in the center of the painting. It is armed with a scythe and is astride an emaciated horse. It is pushing Man into a large box whose door is marked with a cross—clearly a trap door. A hoard of skeletons is invading the scene. They are striking down everyone, with no exception: the king, the mother, and her baby; the knight and the fair ladies; the peasant and the lovers playing music who do not notice the presence of a skeleton behind them. Card players are defending themselves with swords; they have not understood that all resistance is futile. The landscape reflects this death: the trees and grass are dried out, behind the hills there is an infernal fire burning, skeletons everywhere are slaughtering humans: drowning them, hanging them, cutting their throats. A man about to be decapitated is praying in vain, his rosary in his hands. Any promise of redemption or resurrection is absent in this painting. Skeletons are sounding the knell, and there is no hope. See color plates.

Not only the plague provided an opportunity for the creation of a new artistic genre but also, in the nineteenth century, another scourge inundated Europe in waves: cholera.

The art of the Plague mask: Charles de Lorme, first doctor of Louis XIII, imagined the famous protective costume against the plague. "The nose half a foot long, shaped like a beak, filled with perfume with only two holes, one on each side near the nostrils, but that can suffice to breathe and to carry along with the air one breathes the impression of the drugs enclosed further along in the beak. Under the coat we wear boots made in Moroccan leather (goat leather) from the front of the breeches in smooth skin that are attached to said boots and a short-sleeved blouse in smooth skin, the bottom of which is tucked into the breeches. The hat and gloves are also made of the same skin . . . with spectacles over the eyes" [69].

40.1.3 Art at the Service of Medical Science

The Middle Ages placed God in the center of man's preoccupations; already in the later Gothic period, the human being and his representation in art had become closer, more realistic, and more natural. In discovering a creature in the image of the divine, man became the center and the model of artistic creation. Beginning in the fourteenth century in Italy, this new view man had of himself gave birth to the Renaissance during which artists made man the object of their symbolic and esthetic research.

Fig. 40.3. *The Four Horsemen of the Apocalypse* by Albrecht Dürer (1498). Albrect's famous engraving on wood is the fourth of a series of 15 illustrating the revelations of Saint John, who prophesied the end of the world (the series is called *The Apocalypse*). From left to right, the knights represent Death, Famine, Discord (sometimes interpreted as Pestilence), and War. With an angel looking down, the last three are crushing men and women of different social classes with their powerful mounts, whereas Death with the features of an old, gaunt man on his skeletal horse, is casting a bishop into the mouth of a dragon coming out of the bowels of the Earth. This is not a battle scene, but an enraged and ruthless destruction.

In the sixteenth century, the climate of artistic effervescence that marked the Renaissance began to give way to a climate of extreme passions where theological reflection did not exclude massacres, of course, nor did it protect from epidemics. In Western Europe, many artists placed themselves at the service of the Counter Reformation, whereas in Protestant countries, they tended to free themselves from an exclusively religious guardianship.

Seventeenth-century France experienced a different state of affairs, with artistic expression almost entirely devoted to the glorification of the king and civil power.

In the eighteenth century, the role of man in society and in the world became the center of philosophical debates, with artists freeing themselves little by little of ecclesiastic sponsors and throwing themselves into models from classical antiquity.

Fig. 40.4. *Napoleon Visiting the Pesthouse of Jaffa* by Antoine-Jean Gros (1804, Louvre Museum). Bonapart's expedition to the Orient. At the foot of the pyramids, July 21, 1798, Bonaparte conquered Egypt to direct his expedition toward Syria in the first months of 1799 and, with 15,000 soldiers, take El-Arish, Gaza, and lay siege to Jaffa, in the Holy land. In a dreadful and merciless battle, the French troops seize the city; it is a massacre, 2500 prisoners are executed. It is in Jaffa that Bonaparte's soldiers are stricken by an epidemic of the plague that did not relent until the end of the expedition. On August 22, the command of the Orient expedition was given to General Kleber and Bonaparte took to the sea with his best generals, reaching Paris in October 1799. The Egyptian expedition was transformed into a prestigious success by the goods brought back by the members of the expedition: the soldiers' glory, hitherto unseen Egyptian engravings, and the writings of scientists. Antoine-Jean Gros magnifies the Egyptian expedition and represents in *Napoleon Visiting the Pesthouse of Jaffa* a totally imaginary episode of Napoleon's propaganda, when one remembers that when parting from Jaffa, Bonaparte requested that the chief physician, Desgenettes, "put an end to the suffering of our plague victims by giving them opium"; the doctor refused [83]. A.-J. Gros and other renowned painters (David), to serve Bonaparte, and later Napoleon, provided a totally imaginary reading of decisive moments in the history of the Empire, with almost dream-like visions and the use of precise and detailed drawings, undoubtedly intended to make the event represented even more realistic. *Napoleon Visiting the Pesthouse of Jaffa* is an exemplary work.

Thus, from the Renaissance to neoclassicism, the artist emerged as an individual, abandoning the anonymity in which he had enveloped himself during the Middle Ages. From his power to impose ideas and emotions to matter was born the artist's personal responsibility on the work of art that he fashioned with his hands. The Renaissance bloomed in the exceptional atmosphere particular to Italian cities: Citizens took part in this cultural effervescence, and the arts and sciences were protected, as in ancient Athens. The artist played an important role in the sciences: from a professional he became a thinker. The artist acquired the dimension of an individual creator, and not only because he took a place in history, but

he also transformed the history of art into the history of artists and their creations. The artist as citizen expressed his suffering and bore witness to the suffering of others.

An explosion of new artistic genres was seen during this period that was rich in all sorts of discoveries. The many curiosity shops as well as the anatomical and medical treatises of the time oriented some artists toward a naturalistic and descriptive art. The development of the techniques of reproduction such as engraving on leather and lithography also participated in this illustrative art that was accessible to all.

40.1.3.1 The anatomical body as artistic representations

In one of his famous essays, the art historian Erwin Panofsky puts forth the idea that the emergence of anatomy in the sixteenth and seventeenth centuries can only be understood separately from Renaissance art because the history of anatomy is deeply anchored in the history of art. To define the scientific value of the art of anatomy, Panofsky even stated that it should be viewed from an art historian' perspective. In the sixteenth century, the knowledge acquired on the body was represented visually by drawings and engravings made by anatomists and their illustrators. The drawings of Vesale's (1514–1564) *De humani corporis fabrica* (1543) recall the sculptures of ancient Greece, with their clearly defined muscles and wide-shouldered torsos. One of the characteristic aspects of Vésale's engravings is that the dissected organs are framed by living bodies in full health, which averts one's gaze from the dissected organs. The scientific reality of the image is embellished and made more esthetic; the classical conventions of sculpture and painting of the Renaissance determined the elements making up these anatomical representations. Panofsky's idea, wanting the artistic techniques to dominate and model scientific knowledge, is corroborated by Ludmilla Jordanova, who, in a detailed analysis of eighteenth-century wax models, showed how the neoclassical ideas determined the representation of the scientific knowledge of anatomy. The models are perfect specimens of the partially open body, making it possible to see the stomach, the intestines, and the reproductive organs, in a representation where the esthetic norms eclipse a realistic representation.

Lorraine Daston and Peter Galliston, specialists in nineteenth-century medical representations of the body, speak of the continual struggle between scientific objectivity and artistic subjectivity. In the nineteenth century with the new techniques of representation, scientists hoped to eliminate any "artistic contamination," and the new tools (e.g., photography, radiography) would eliminate the subjectivity of the artists. However, Daston and Galliston showed that the introduction of mechanical printing "neither created nor closed the debate on how to represent (the body)," and the replacement of engraving by photomechanical instruments did not end the interpretation; the very presence of the photographer meant that these photos were made through mediation. The new tools attenuated the illusion of perfect transparency while advancing the concept of an objectivity accomplished through a mechanical reproduction.

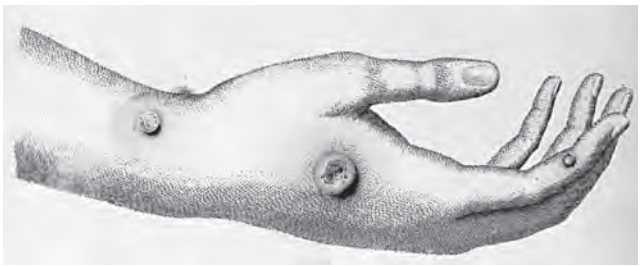

Fig. 40.5. William Skelton (eighteenth century) engraving on leather, 70 × 180 mm. Hand of Sarah Nelms, who had contracted cowpox milking cows with the disease.

Anatomical objects, bodies, and models have aroused the interest and the curiosity of the public since the end of the fifteenth century. It was not until the end of the eighteenth century that it disappeared behind the hospital walls. In some parts of Europe, exhibiting human organs remained an attraction of country fairs until the beginning of the twentieth century. From the anatomical theaters of the sixteenth century to today's anatomical collections, anatomical objects have progressively been integrated into a context of medical-scientific museums.

40.1.3.2 Wax anatomical models

Throughout history, anatomists have attempted to reconcile the contradictory demands of authenticity and didactics in their teaching of medical knowledge. From Vésale to von Hagens, anatomists have been torn between the desire to preserve the authenticity of the human body and the educational advantages of models. At the beginning of the Renaissance, observing an anatomist carrying out a dissection was the only means for future physicians and artists to acquire a sense of the inside of the human body. When the Belgian anatomist Andrea Vésale in Bologna where his colleagues Jacobus Sylvius of Paris, and later Nicolas Tulp in Amsterdam, carried out public dissections, neither their students nor the spectators were authorized to touch the body. The cadavers decomposed rapidly, to such an extent that the dissections had to be done rapidly and by experts. The need to preserve the bodies, as well as the desire to set off specific physiological characteristics, stimulated the invention of better preservation techniques. From the beginning of the twelfth century to the sixteenth century, many embalming and preservation techniques were experimented. The Dutch anatomist Frederick Ruysch (1658–1731), the successor of the illustrious Tulp, developed unprecedented cadaver preservation and presentation techniques and made possible the emergence of a new type of anatomical artifact: The cadaver became a work of art more than a scientific object.

Ludmilla Jordanova is a professor of visual arts at the University of East Anglia (UK) specialized in visual culture of the eighteenth and nineteenth centuries, cultural history, portraiture, style, historiography, gender and kinship, and science and medicine as forms of culture, she highlighted that the contradictory demands of authenticity and didactics

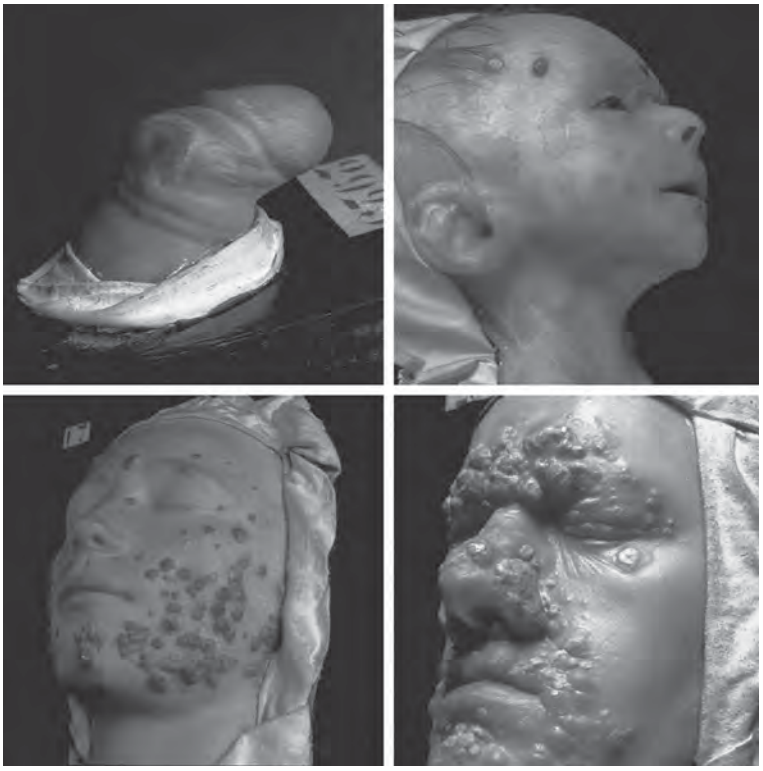

Fig. 40.6. Wax sculpture (Jules Baretta (1834–1923) Moulages 2923, 11, 17, 208 Jules Baretta (1834–1923) Collection générale de l'Hôpital Saint Louis. Extrait de: Le musée des moulages de l'hôpital Saint-Louis. G. Tilles et D. Wallach, 1996. Ed. APHP et Doin. See color plates.

repeatedly appeared through the history of anatomical artifacts. After the Renaissance, medical education increasingly meant allowing students to practice on anatomical bodies. The increasing demand for cadavers, combined with the emergence of stricter laws on obtaining them, forced anatomists to search for substitutes. Although in the seventeenth and eighteenth centuries, the shortage of cadavers led to the creation of false bodies, the development of wax models, produced to respond to these educational demands, was an alternative that had its advantages. Beeswax had the advantage of both resembling the organic texture and being malleable. Between 1750 and the end of the century, some sculptors of Bologna, such as Lelli and Morandi, and the Florentine masters Caldani, Fontana, and Piranèse, raised wax sculpture to the status of art; their models thus went beyond the clinical education context to private collections, and later to museums where they can be admired today.

40.1.4 Tuberculosis, Self-Portraits of the Diseased

> Without fear, and without disease, my life would be like a boat without oars. Edward Munch [6]

Tuberculosis is emblematic of the close relation between art and disease. It is the mark of the Romantic Movement. The most reputed masters of medicine of the time did not immediately see the infectious and contagious character of tuberculosis; for years they attributed to the disease characteristics that were almost charming and intoxicating for the Romantic creative minds of the time. Children and young adults infected with tuberculosis were described with a wealth of details: "slender and delicate beings, with fine traits, beautiful hair, with little physical activity, but with a lively intelligence and early maturity, gifted in the arts and endowed with a particular aptitude to understand and feel" [98].

According to Laennec, the ill, belonging to this morphotype "seem to owe the origin of their illness to sorrow." "Among the occasional causes of consumption, I know no other more certain than the sorrowful passions when they are profound and long-lasting," [10] the doctor asserted. There were many writers, poets, painters, and musicians stricken by consumption or who saw their loved ones waste away from this disease, and who colored their works with these romantic and moving attributes [16].

The birth of painting was reduced to the vision of Narcissus contemplating his reflection in his mirror by Leone Batista Alberti [1], which lies directly within the self-portrait. Every great painter left at least one self-portrait, particularly the expressionists of the beginning of the twentieth century, perhaps influenced by the self-portraits of Van Gogh. In particular, the self-portraits of Munch, Schiele, Beckmann, and Kokoschka must be remembered. The portrait, and more specifically the self-portrait, was the preferred subject of the Romantics and the Expressionists. Illustrating both physical and psychological torments, troublingly intimate self-portraits

were surrendered by young artists suffering from tuberculosis. But tuberculosis, even though it was the emblematic disease of the time, was not the only one to inspire the morbidity of a great number of works of art of the nineteenth and twentieth centuries. Other diseases such as syphilis and the Spanish flu took many artists. The Great War menacing on the horizon also certainly caused a great deal of anxiety. Freud's studies on psychoanalysis and the unconscious also motivated the introspection that was in the air, favoring the pathos released by certain works of art. Artists no longer painted the body as it was, in the style of the realists, but rather desperately tried to give it a psychological dimension, incessantly searching for new means of expression to translate the extent and complexity of human suffering.

Edward Munch. Munch's work oscillates between illness and Puritanism from its very beginning. In the childhood and youth of this painter, the encounters with illness and death were decisive experiences. He was profoundly marked by the tuberculosis of his sister Sophie, who was not yet 15 years old. Fully aware of what was happening, Munch, younger by a year, followed the progression of this disease and his sister's drawn-out death. In 1885–1886, he formulated this experience in a pictorial mode, through *The Sick Girl*. At the age of 5 years, he had already lost his mother, taken by this disease present throughout all levels of society, still an epidemic 100 years ago. In 1889, during a voyage to Paris, the artist's father also died.

Given these circumstances, there is nothing surprising about the first of Munch's works being devoted to this subject. Much later, toward 1930, Munch again turned his attention to this painting and wrote to the director of the Oslo Nasjonalgalleriet: "Concerning *The Sick Girl*, it is true that we live at a time that I call the pillow epoch. Many were the painters who represented ill children on a background of pillows . . ." Among them, we must cite Christian Krohg (1852–1925), whom Munch deeply admired, and Hans Heyerdahls (1857–1913), whose technique is perhaps the closest to Munch's. In 1880–1881, Krohg had painted *The Sick Girl*; Heyerdahls was the author of *Child on the Point of Death*.

The genesis of these three works presents great similarities: All three painters had to work the death of their own sisters through a pictorial mode. The Swede Ernest Josephson (1852–1906) and the Dane Michael Ancher (1849–1926) can also be placed among those that Munch called the pillow painters.

Krohg's painting of a sick girl has something monumental about it: Sitting in a rocking chair, she is presented facing the spectator. In the highly nuanced whites of the painted surfaces, the square form of the pillow framing the head and the bust take on a symbolic meaning. A form inherited from the pathos of the Middle Ages, the square halo—the sign of the saints canonized during their lifetime—complete the symbolic importance of the roses losing their leaves and echoes the ephemeral side of earthly existence: The existence that the sick child holds between her entwined fingers, and that she presents to us. Krohg makes a memorial of the portrait of his young sister, who stares at the spectator with her large, open eyes full of fear. Other variations on the same theme assume, on the contrary, an air of reflection on a naturalist motif: bouquets of flowers, the drape of blankets, or the light reflected by the small vials that preoccupied the painter.

Fig. 40.7. *The Sick Girl* (Christian Krogh, 1880–1881) Huile sur toile, 102 × 58 cm Oslo, Nasjonalgalleriet.

It is an entirely different story with Munch's painting entitled *The Sick Child*, reworked in the 1890s. Today it is difficult to understand the hostility and indignation that this painting set off when it was presented to the public for the first time in October 1886, during the autumn exhibition of the Christiana artists. The theme was quite conventional, but the reception was indignant—especially in the artistic milieu—because of the impudence of its author who dared exhibit a painting whose main elements recalled a poorly ordered sketch, with traces of the preparative work still visible. Here we are presented with a painting whose power is fully interior. The painter having been touched to his most profound being by tuberculosis, losing two loved ones, his pictorial search was oriented beyond realism, reigning at the time, to plunge into the meanders of his most intimate emotions.

Christian Krohg, who was also a journalist, wrote to defend his admired colleague:

> He paints, or more precisely he observes objects differently than other artists do. He sees only what is essential, and sees it naturally, painting nothing but that. This is why in general Munch's paintings are not completed, as people so readily note. But yes they are! They are indeed completed: his work in all its culmination. Art is completed when the artist has truly said everything in his heart. . . .

Fig. 40.8. By the Deathbed (E. Munch, 1895) oil on canvas, 90 × 120.5 cm; Bergen, Collection Rasmus Meyer.

This is indeed a new form of expression opening to this young generation of avant-garde artists of the Christiana bohemia. Perhaps the premises of abstract art. In any case, it was a way to reach out toward the materialization of abstract things such as disease, death, but the painter's emotions also, when faced with such emotions. Munch noted

> In fact, my art is a confession that I make of my own free will, an attempt to elucidate, for myself and my relation to life. ... At the heart, it is a form of egotism, but I do not relinquish hope that it will help me succeed in helping others to understand.

Munch grew up in a very Catholic tradition, and his words can be heard in this sense, almost like the words of a martyr, suffering to free other human beings from their sins. Munch's sufferings were his loyal companions throughout his life, his inspirational Muses: he explored them, confronted them, and finally attempted to channel them into his art.

As if incessantly calling suffering and death to himself through his works, Munch was himself the victim of another illness: the Spanish flu. He survived but it took him some time to recover, which gave birth to an entire series of self-portraits in 1918–1919. The characteristics of these canvases are interior tension and isolation. An instrument of merciless self-examination, Munch's self-portraits accompanied his entire artistic creation, from their very beginnings until his last breath.

With the display of the most splendid colors, Munch shows the immense difficulties he had in overcoming disease (self-portrait after the Spanish Flu, 1919). In 1940, 4 years before passing away at the age of 81, in his Ekely home, Munch painted self-portraits that showed a man who knew himself to be between life and death. The two paintings that he worked on until the end of his life are the large-format self-portrait: between clock and bed, and an oblong painting of a slightly smaller size: self-portrait by the window. The vertical structure of the first painting in an oblong format expresses the opposition between the standing and lying position. A human being's life is no more than a brief victory over gravity and matter. He pulls only himself up to his full height to lie down more fully. His life becomes the painting, which illustrates this victory [6].

Amadeo Modigliani. On January 24, 1920, Amadeo Modigliani succumbed to tuberculosis meningitis. Jeanne, his wife, 8 months pregnant, committed suicide the next day. Modigliani was buried on January 27 at the Père Lachaise cemetery in Paris; Jeanne Hébuterne's body rests at his side. This tragic double death made Modigliani an emblem of the Romantic bohemian artist brought down by tuberculosis.

Although he does not illustrate disease directly, Modigliani's work would undoubtedly be entirely different if, from a very young age, he had not seen death at close hand. In 1901, at only 17 years of age, Modigliani contracted a respiratory disease that weakened him throughout his life. Born in Italy in 1884, he studied art in Florence and settled in Paris in 1906. There he mixed with avant-garde artists: Pablo Picasso and Constantin Brancusi were among the most influential figures in his art. He was greatly inspired by fauvism, a movement that favored pure color as a mediator of emotions, at the expense of figurative realism. Like many artists of the time, his research was oriented toward the strictest representation of lines and shapes, made in the image of African masks. Modigliani's work, almost exclusively portraits, is characterized by simple lines, strong colors, and his highly stylized manner of painting eyes in a single color, giving the characters represented a feeling of "presence absence" close to death.

When death lies in wait, the condemned artist must urgently synthesize his work and research: making dazzling bounds in the history of art, he delivers modernity. If Modigliani's portraits approach the purity and simplicity of certain icons, it is because he knew how to perceive the divine in his models, beyond death. Illness restores the attachment to life and meaning. In this sense, Modigliani's portraits are hymns to life and the beauty of the human being. The characters he painted have a strong presence but seem to escape humanity: they have essence. Modigliani painted life, with the wisdom of someone who had befriended death.

40.1.4.1 Egon Schiele and Gustave Klimt during the Spanish Flu

In the autumn of 1918, at the close of the First World War, another, even more deadly disaster was in preparation. A viral disease that would soon be called Spanish flu was about to kill millions of people in just a few months, and then disappear without a trace. We saw above that it touched Munch, who survived its effects. To the sad list of victims appear the illustrious names of Klimt and Egon Schiele (another example can be added, one of many others, the poet Guillaume Apollinaire).

Egon Schiele was born in 1890 in Tulln, a small town near Vienna in Austria, where he died in 1918. From childhood, he expressed a true talent for drawing. His father, a station-master, encouraged him in this endeavor, but afflicted with a

mental illness, he died in 1905. This early death tarnished Egon Schiele's youth and gave him a vision of the world that from then on would often be dark and tortuous.

Schiele is a rebellious, avant-garde artist. He was very young when he left the Vienna School of Fine Arts, too academic for his taste, and in 1909 created "the group for Nouveau Art" with a few friends. His first works were inspired by Impressionism, but very quickly he was attracted by the Viennese Secession. His work was highly influenced by the work of Gustav Klimt. However, other influences such as Van Gogh, Hodler, and Georges Minne also play a primordial role in the evolution and construction of his style. It is in the 1910s that he began to assert a more personal style characterized by sparseness of form, sobriety of content, and the use of backgrounds without adornment, with the character or subject detached from this background. Schiele attached great importance to self-portraits. He attempts to transcribe the anguished interiority of the ego by excentric positions of the body or the hands that he paints. Although Schiele's paintings do not have disease as a subject, these portraits are the image of bodies conscious of their biological limits. He contributed to retranscribing a vision of his reality with no make-up or artifice, exacerbating the suffering and anguish of this turning point in history. The number of Egon Schiele's self-portraits deserves interest, because he is the only one to have gone so far into exhibitionism, representing himself nude many times: full-face, in profile, kneeling, sometimes masturbating or, in *Eros* (1911), brandishing his erect, reddened penis. The young painter undoubtedly rubbed shoulders with disease when he had prostitutes pose for him. Syphilis is hinted at in these bodies between life and death, with bluish, greenish, and sometimes muddy-colored flesh, where only the sex takes on a reddish hue. We feel a dread associated with sexuality. Is it psychological, psychiatric, or rather is it the portrait of a venereal disease that Schiele is providing here?

Gustav Klimt, born in Vienna in 1862, at first takes up decorating walls for public buildings, painted in the great academic tradition, with a pronounced taste for naturalist detail. The symbolic cycle of the University of Vienna ceiling, *Philosophy, Medecine and Jurisprudence* (1900–1902, destroyed in 1945), prompted a controversy because of the crudeness of its eroticism and the asymmetric part of its composition. Gustav Klimt founded the Secession group, which advocated a renaissance of the arts against the moral order. The magazine *Ver Sacrum* (Sacred Spring), as part of its policy, was the theoretical support in all fields of application. Klimt's style made use of decorative resources: solid-color surfaces treated in the painting's one-dimensional plane, the snaking lines strictly delimiting the motifs, an abundance of adornments and precious materials. From these highly decorative colored backgrounds spring out the figures, lascivious and organic. Whether it be symbolic, naturalist, or architectural, Klimt's work always treats the relations between eroticism and death; it draws the frontiers of the indecent and the morbid. On February 6, 1918, Klimt died following a stroke resulting from the flu in Vienna. Egon Schiele, his student and also taken by the flu at the end of the year, made his portrait on his deathbed.

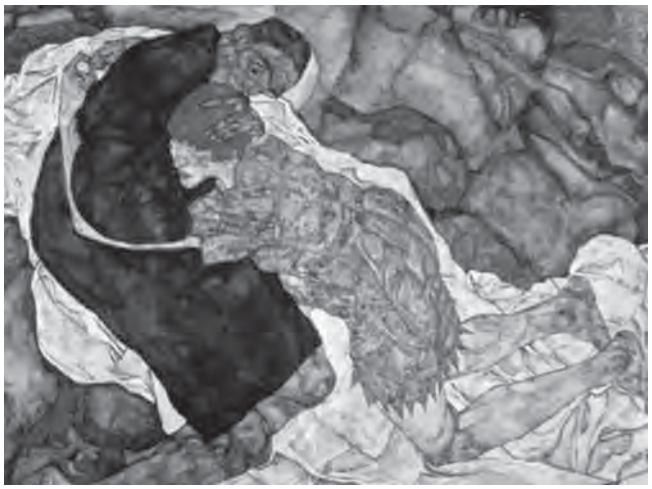

Fig. 40.9. Death and the girl (E. Shiele, 1915).

Fig. 40.10. *Death and Life* (G. Klimt 1916) oil on canvas, 178 × 198 cm Vienna, Dr Rudolf Leopold Collection. See color plates.

40.1.5 The Syphilitic Female Model: A Turning Point in Art History

Very early on, women were identified as evil temptresses in the image of Eve, at the origin of original sin. Woman as the symbol of evil is a widespread theme in the history of art, under a variety of forms. This collective and ancestral imagination was continually updated with sexually transmitted diseases such as syphilis.

In the nineteenth century, the representation of the nude brought about the beginnings of a pictorial revolution led

principally by Courbet and Manet. Courbet rejected academic painting with its smooth, idealized nudes, but he also directly attacked the hypocritical decorum of the Second Empire, where eroticism, or even pornography, was tolerated as long as it portrayed mythological or dream-like painting. The realism of Courbet, who later stated that he had never lied in his painting, continuously pushed back the limits of what was considered presentable. With *Origin of the World*, he exhibited, as it were, the hidden part of Manet's *Olympia*. Courbet and Manet introduced the idea of painting the female body unveiled, insisting on what was uncomfortable to show in the female body in the culture of the time. The painting of "*Origin of the World*" by Manet figuring a detailed and "vivid portrait" of a woman sex was a command of a private commission from a wealthy collector which remained a secret. After being bought by another private collector it remained concealed to visitors by a painted flap covering it. Twenty years later, the public discovered the painting, which was widely commented on in the press. Today it is on public view at the Orsay Museum in Paris. This stage in the history of art, which was in many ways remarkable, allowed syphilis to appear in early twentieth century paintings.

> . . . Teach people true history by showing them true painting, . . . by true history I mean history free from superhuman interference, which from time to time has perverted and overwhelmed the individual. By true history I suggest that which escapes the yoke of any fiction. In order to paint truly, the artist must keep an open eye on the present, he must see with his eyes and not with the back of his neck, . . . be able to portray the morals, the ideas, the aspect of my time, according to my appreciation; to be not only a painter, but also a man; in a nutshell: to create living art, that is my aim [22].

Under the Second Empire, in the name of good taste, David's theories, as taken up by Ingres were revived. The criteria for appreciation of most of the well-to-do classes as well as the Fine Arts Academy were the pleasantness and sumptuousness of the work. A return to overwhelming classicism emerged and rejected new trends such as realism and romanticism. The word "realism" appears in a text by the critic Gustave Planche [56], who contrasted the realist manner with that of supporters of ideal beauty. This term designates more precisely a movement that, between roughly 1848 and 1860, reacted against backward-looking academicism and neoclassicism. The limits of the movement remain vague in time and space as, although it was originally a French movement, realism fed on Dutch, Spanish, and English contributions, and spread throughout Europe.

From the meticulous and cold technique that was typical of neoclassicism, a cruder, more spirited method developed, often closer to sketching. Another, more radical change also took place in the choice of subjects. In the classical hierarchy of genre, noble historical painting stood in first place. In reaction to this, the realists depicted scenes of everyday life. Academic painters referred to this subject matter as "ignoble and impious caricatures."

In 1863, at the behest of Napoleon III, a "Salon des Refusés" opened, in opposition to the academic salon that was held each year for 8 years at the Palais de l'Industrie.

This "Salon des Refusés" was the cradle of modern art, intending to paint life as it was by getting rid of eclectic and complicated backgrounds, as well as the conservative bourgeois morality of the time. At this turning point in the history of art, such scandalous paintings as Courbet's *Origin of the World* (not exhibited in public: it was a commission by a foreign "art-lover.") or Manet's *Luncheon on the Grass* appeared. These two key paintings were to open the door to a new form of expression that touched on cruder, more taboo subjects, dealing with the most daring avant-garde topics. As a reaction to classical female models, who could only be depicted in the nude if they had the status of goddesses, hence belonging to the supernatural, the younger artists were tempted to humanize these women, going so far as to show their most disturbing shortcomings. Prostitutes being the anti-goddesses of the Christian morality in force at the time, it was only a small step before the young avant-gardists chose them as their favorite models.

> Venereal disease was the great fear of the nineteenth century. As it struck sexual relations occurring outside the sterile confines of married life, in the same way as HIV-AIDS does today, syphilis testified to the guilt of pure carnal pleasure, a mortiferus pleasure. Bleak romanticism was to make syphilis quite a singular affliction [74].

Alphonse Daudet's son, who had become a doctor, professed

> The germ of this terrible affliction, treponema, as it should be given its proper name, is both the whip of genius and talent, heroism and spirit, as well as that of general paralysis, tabes—Neurological attack—and of almost all degeneration (. . .)
>
> Hereditary treponema, strengthened by interbreeding between syphilitic families, has and will play a role comparable to that of the "fate" of antiquity. It is a personality, invisible but present, which moves romantics and unbalanced persons, sublimely beautiful deviants, pedantic or violent revolutionaries. It is the ferment which raises the slightly heavy dough of peasant blood and refines it within two generations. It turns the son of a maid into a great poet, a peaceful middle-class man into a satyr, a shopkeeper into a metaphysicist, a sailor into an astronomer, or an astronomer into a conqueror [24].
>
> At the end of the nineteenth century, it was not long before syphilis was given an esthetic, that of the awful and decadent seduction. Beneath the ardent and coveted flesh of women lay syphilitic death, crouching in the depths of the vagina like a moray eel in the depths of a hole, lying in wait for the foolhardy, fascinating him. It was in the pleasure dens of Montmartre that many artists sought inspiration from these venomous and disturbing creatures [78].

Pablo Picasso

> We Spanish, we go to Mass in the morning, the bullring in the afternoon, and the whorehouse in the evening [70].

Picasso, who was famous among other things for his sexual ardor, did not escape the venereal disease of the century. Like

the other young avant-garde artists of the time, he was a regular at brothels, where he liked to paint young prostitutes and ball scenes. It was in 1901, during a trip to his native Spain, where he contracted venereal disease. It was a severe blow and threw Picasso into a deep depression. On his return to Paris, he consulted Dr. Julien, a venereologist, who was also the doctor for the woman's prison at Saint Lazare. Picasso requested permission to visit the prison hospital, in the aim, as he was to explain later, of finding cheap models. The doctor opened the doors to him and guided him through the wards.

> It was a sinister building: the prostitutes infected with or suspected of having venereal disease were imprisoned, on foot of a simple administrative decision, along with rebellious women arrested for soliciting on the public thoroughfare. The Sisters of Saint Joseph dressed in black and blue dispensed care there and ruled the wards with iron discipline. The place was appalling and hopeless. The inmates were permitted to keep their child until it was weaned. The impression made on the young painter was horrifying. The nightmarish vision of these women, wandering about in silence, as imposed by discipline, was to become the main influence of Picasso's famous blue period. The dark indigo shifts in which they were dressed, and the more or less Phrygian caps that were used to indicate those infected with syphilis, made a strong impression on his imagination, as did the prostitute mothers, protecting their newborns in the folds of their uniforms to guard them from the icy cold of the gloomy corridors. On returning to his studio, he recorded his emotions, keeping mainly to images of motherhood and the drawing of the white caps.
>
> "I want to do a painting of the drawing I'm sending you, *Two Sisters*. The painting is that of a whore from St Lazare and a mother," he wrote to Max Jacob in 1902.
>
> Hence, the blue period was born of venereal disease [72].

From 1904, Picasso recovered from his depression and went on to quite a different style. The pink period with its harlequins got the painter back on his feet again, and in 1906 he returned in strength to the world of brothels. Rejecting all the conventions of the time, quartering the shapes of five women around a bowl of fruit, Picasso composed his first canvas of exorcism: *Les Demoiselles d'Avignon*. On being introduced to the large, unfinished canvas, which remained hidden from public view for a long time, Max Jacob exclaimed "It's the introduction of syphilis to painting" [73].

Toulouse-Lautrec. It is in the very essence of his tortuous lines, set out almost organically, that Toulouse-Lautrec inhabits his paintings of women haunted by evil. From them exudes an uncommon humanity, their looks are present, their postures almost disturbingly natural [79].

> It was in the brothel at 6, rue des Moulins that in 1894 Toulouse-Lautrec set up camp, as he liked to say. The painter was fond of venal women, without doubt. Much has been said about Toulouse-Lautrec's stays in brothels. A legend has grown up, fed by Thadée Natanson, the director of the Revue Blanche, which left us with a portrait of Lautrec as an alcoholic, crazy about painting and women, seeking the intimacy of a family in brothels.

Fig. 40.11. *Les Demoiselles d'Avignon*. Pablo Picasso, 1907. Museum of Modern Art, New York. Oil on canvas (224 × 234 cm). See color plates.

> In snatches, as he sharpened his pencil, says Yvette Guilbert, he told me of his liking for life in a brothel, watching the throb of prostitution, seeing modesty falter, penetrating the sentimental pain of the poor creatures, the civil servants of love. He was their friend, sometimes their advisor, their brother in pity. When he spoke of these poor women, minute by minute, the emotion in his voice betrayed the warm pity of his heart. . . to the extent that I often wondered if Lautrec did not find a mission of beauty in his willingness for fraternal and Christian commiseration toward these women stripped of modest pride (Yvette Guilbert) [40].

It was through truth that Toulouse-Lautrec supported these women; he painted them as he saw them, directly. His sketches are of a marvelous exactitude in their cruelty, as a critic of the time said of the series of lithographies *Elles*. Art is not there to turn us away from life; on the contrary, it brings us back to life through sensitivity. The work of the artist is to portray through this sensitivity what is vibrant and pathetic in life. In the hopeless brothel, purged of any illusion, Lautrec pays homage, to reality.

40.1.5.1 AIDS and contemporary art AIDS arrived in the artistic iconography of the twentieth century in a movement that could be qualified as contemporary romanticism, where the figure of the artist and his torments takes a central place in the work of art. In the liberal society of our time, AIDS is associated with the great moral debates around homosexuality and drugs and is often the occasion for strong demands. As it was identified around 1980, AIDS has closely accompanied the contemporary avant-guard artistic scene, in particular painting and images. This devastating epidemic is

Fig. 40.12. *Woman Putting on Her Stocking*, by H. de Toulouse-Lautrec (1864–1901) (domaine publique). See color plates.

no longer simply a subject of inspiration for artists, but rather has become an open door to a political and social art, controversial. Just as tuberculosis was the mark of Romantic artists and then bohemian artists, AIDS haunts the underground world of art of the 1980s. The young New York artists, gravitating around the figure of Andy Warhol, contributed to the explosive coming-out of American society's unspoken secrets concerning homosexuality, drugs, and AIDS, which were, at the time, intimately entwined. Indeed, it remains under the aegis of religion that Protestant and puritanical American society perceives this disease as unavoidably linked to sin.

The many celebrities who were killed by this disease gave AIDS an uncommon currency and production in all genres, whether through the visual arts, music, film, literature, folk art (graffiti, posters, etc.), and crafts.

Artistic production directly inspired by AIDS took root in the United States. This disease raises not only health problems but also, much more broadly, moral and political issues. Most of the American artists between 1980 and 1995 pay homage in their own way to those touched by AIDS. Among the most famous that can be mentioned is Andy Warhol, who was the central figure of the pop art movement that inspired so many artists of this generation, as well as Keith Haring, Nan Goldin, Felix Gonzales Torres, Robert Mapplethorpe, Duane Michals, and Franck Moore, to cite only the most important figures.

From rage to activism, from the metaphor to the memorial, their diverse artistic approaches reflect the complex way that the epidemic moved and continues to move contemporary America. The art that developed around AIDS is the reflection of many and various demands, and provided the opportunity for many provocative exhibitions on this theme. Today it has gone beyond the Western world and shows up particularly in Africa, the most highly affected continent, and most specifically in Southern Africa where popular expression is extremely rich [30].

The exhibition *From Media to Metaphor: Art about AIDS* organized in 1992, by R. Atkins and T.W. Sokolowski, brought together a large number of works around the AIDS theme. This great exhibition took a fresh look at the traditional gallery space, transforming it into a true forum for discussion and protests, uniting in a pot-pourri of photographers, painters, sculptors, and also activist groups that had launched into the AIDS theme. The epicenter of the HIV epidemic originally stemmed from the homosexual populations on the east and west coasts of the United States, before

Fig. 40.13. Gran Fury, "Read My Lips" (Boys) [1988] posters produced by the group Act Up. An act up appropriation of a World War II year photograph of two sailors kissing. rpt. in Crimp and Rolston [56].

spreading to other social groups—Gmerk Mirko dans son "Histoire du Sida": montre que les trois foyers homosexuels d'origine ont été New York, Los Angeles et Paris—[41]. The initial artistic response to AIDS was a response by activist artists of the homosexual community. Some in the United States, and even sometimes the authorities, referred to AIDS as the gay plague or even WOG (Wrath of God). The activist groups formed first in response to this growing homophobia before the populations affected by AIDS.

In the 1980s, the activist group Act Up began to write informative material on the risks of AIDS, published in New York periodicals, and then launched a quasi-publicity campaign, covering the walls of the city with all sorts of tracts, posters, and stickers, made up of shock images, warning against the dangers or the epidemic. Among the most widespread posters were *Silence = Death* or *Fight Homophobia, Fight AIDS*. This group, created in 1987, spread across the globe in a mere 5 years, forming a dozen groups in other countries. Gran Fury is a group associated with Act Up that makes immense politically committed posters.

> One of their major pieces is a monumental black and white poster. The image depicts two government officials shaking hands. What is disturbing is the angle of the photo: the camera is beneath the men, looking up to imply that they, in their position of power, tower over the people. Text stating: When a government turns its back on its people, is it civil war? [89]

This multiform art is highly accessible and has been brought together for the first time by Keith Haring, who was inspired by the serial art of Andy Warhol, opening a boutique in Soho in New York City in 1986, where he sold posters, t-shirts, pins, his stylized characters, illustrating simple concepts simples that appealed to a wide audience—life, death, love, and sex—which became very popular. He opted for an art that was accessible to everyone, meaningful and direct.

> Throughout his career, Haring devoted much of his time to public works, which often carried social messages. He produced more than 50 public artworks between 1982 and 1989, in dozens of cities around the world, many of which were created for charities, hospitals, children's day care centers and orphanages. (…); a mural on the exterior of Necker Children's Hospital in Paris, France in 1987. (….)
>
> Haring was diagnosed with AIDS in 1988. In 1989, he established the Keith Haring Foundation, its mandate being to provide funding and imagery to AIDS organizations and children's programs, and to expand the audience for Haring's work through exhibitions, publications and the licensing of his images. Haring enlisted his imagery during the last years of his life to speak about his own illness and generate activism and awareness about AIDS. (….)
>
> Keith Harring died of AIDS-related complications at the age of 31 on February 16, 1990. A memorial service was held on May 4, 1990 at the Cathedral of St. John the Divine in New York City, with over 1,000 people in attendance [53].
> (….)
>
> There can be no doubt that the artist's battle with AIDS had a profound effect on his artistic vision. 'To live with a fatal disease,' he confided to his biographer John Gruen shortly before his death, 'gives you a whole new perspective on life.' The resulting pain and anguish are eloquently expressed in Haring's two collaborations with William Burroughs: *Apocalypse* (1988) and *The Valley* (1989).
> (….)
>
> The true 'horror of AIDS had come to light' for Haring in 1985, and he had for some time regarded himself as a prime AIDS 'candidate'—even before discovering the first Karposi sarcoma on his leg during a trip to Japan in 1988. Not only had numerous intimate acquaintances, including his ex-lover Juan Dubose, already succumbed to the disease. Rumors of Haring's own infection were rife long before he himself learned that he was HIV-positive. More than a year before the diagnosis, *Newsweek* had tracked the artist down in Europe to ask if his protracted stay there was a cover-up for his affliction with AIDS [36].

Felix Gonzalez Torres is a conceptual artist from Cuba. He made a substantial contribution to the dialogue on homosexuality and AIDS. He was himself killed by the disease after having seen many of his companions and friends being taken. His works are of an extreme formal simplicity but still bridge many emotions. He is also a partisan of an art based on an exchange with the spectator in which the latter takes part in the evolution of the work. In *Lover Boys*, he exposes a simple pile of monochromatic candies weighing 355 lb, the exact combined weight of Torres and his partner. Visitors are encouraged to take candies and eat them. It is a sort of conceptual self-portrait that Torres provides, the scattering of the body until it totally

Fig. 40.14. Untitled (Keith Haring. 1984) acryclic on muslin tarpaulin 120 × 180 in private collection. See color plates.

disappears. The love between the two bodies symbolized by this uniform and indistinct pile of candies. This idea to also include the notion of weight seems directly related to the fact that the first cases of AIDS, with no access to triple therapy at the time (or before it was perfected), suffered from a spectacular weight loss as they approached death. Also, the fact that the visitors are encouraged to eat the candies, placed on the floor, symbolizes the acceptance of the disease by others. With very simple means, Torres succeeds in expressing the emotions a young man afflicted and contaminated by AIDS goes through at the dawn of the twenty-first century.

Fig. 40.15. Untitled (Felix Gonzales Torres. 1993). This short biography by the artist himself is very representative of the Torres's work and life, simple but a bridge of multiple emotions.

> 1957 born in Guaimaro, Cuba, the third of what would eventually be four children 1964 Dad bought me a set of watercolors and gave me my first cat 1971 sent to Spain with my sister Gloria, (….) 1985 first trip to Europe, (…) 1986 Mother died of leukemia 1990 Myriam died 1991 Ross died of AIDS, Dad died three weeks later, a hundred small yellow envelopes of my lover's ashes-his last will 1991 Jorge stopped talking to me, I'm lost—Claudio and Miami Beach saved me 1992 Jeff died of AIDS 1990 silver ocean in San Francisco (….) 1991 went back to L.A., hospitalized for 10 days (….) [39]

These exhibitions, whose central theme was AIDS, were designed to awaken the authorities and the public and encourage them to turn their attentions to the problems caused by AIDS, though certain people seemed in favor of the disease. As Vivien Raynor commented in a *New York Times* article on September 23, 1990:

> This is a forum for pleading many causes, notably that of sexual freedom, and there is a strong sense of paranoia and self pity. It is as if the plague that directly or indirectly threatens everyone is somebody else's fault, and to the degree that doctors have failed to find a cure, it is. But like artists-against-the-bomb shows, this one seems concerned primarily with going on record against AIDS, as if there were a party in favor of the disease.

Faced with a new evil, the old reflexes are awakened: as during the great plague epidemics, confronted with such deadly scourges, people sought scapegoats, and, for some, this disease was experienced as punishment for sin. The first reactions are to point to the minority groups who were the first victims. But soon it was clear that AIDS affected not only homosexuals and drug addicts but all levels of society. Beginning in 1986, heterosexual cases appeared, as did cases by blood transfusion or by contamination from mother to baby. Artists such as Jackie Kirk worked on the theme of the changing face of AIDS. She painted protraits at the beginning of the 1990s of AIDS patients from diverse social and ethnic backgrounds, showing that the "gay plague" had made room for a scourge that could reach everyone, one that had to be attacked together.

40.1.6 Images of Disease: Photography and Photographers

The history of the beginnings of photography retains the names of several poineers: Thomas Wedgwood (1771–1805) in 1802 managed to obtain fleeting photograms, images of objects placed on paper coated with silver chloride that turned black when exposed to light. On May 5, 1816, Nicéphore Nièpce (1765–1833) was the first to succeed in producing the first negative on paper, but since it was not fixed, it blackened when exposed to light. In 1822 in a darkroom, he exposed a tin plate covered with a mixture of nitric acid used by engravers for etchings, whose photosensitive properties and lavender essence he had discovered. He also obtained, without drawing, painting, or engraving, the first photographic image (the heliotype): "the standing table." This is not yet photography (or heliography) and the image is a

"point of view." In 1826, Louis Jacques Mandé Daguerre (1787–1853), owner of a street show using animated images created by lighting effects, perfected Nièpce's discovery and succeeded in convincing François Arago (1786–1853), Secretary of the Academy of Sciences and Director of the Paris Observatory, of the advantages of the invention. At the Academy of Sciences session on January 8, 1839, it is said "that France then nobly endows the entire world with a discovery that can greatly contribute to the progress of arts and sciences." Henry Fox Talbot (1800–1877) reinvents, like Nièpce before him, the negative paper but pursues his invention and obtains a positive image in less than a minute of exposure and can be credited as the true inventor of today's photography. He calls his images kalotypes (kalos = beauty). Invention communicated to the Paris Academy of Sciences in 1839, it was eclipsed by Daguerre's invention, a positive image obtained more directly, one that was more meaningful to the public. Hippolyte Bayard (1801–1877), of all these men the least well-known, as early as February 1839 invented a process to obtain positive images directly on paper. Finally, also in 1839, John Herschel (1792–1871), an English astronomer, invented the words "photography" (written with light), "negative," and "positive."

The use of photography in medicine developed rapidly in the beginning of 1840 and a few photographers even seemed to think that the image could provide details beyond the real, such as the "optogrammes" imagined by pathologists persuaded that in the eyes of the murdered they would find the image of the assassin still fixed on the retina, or even in the work of Doctor Baraduc who, holding a photographic plate over a sedated patient, thought he could obtain the "aura of his nightmare."

The first medical daguerreotypes were microphotographic images made in Paris in the beginning of 1840 by Alfred Donné, giving a course in microscopy at the Paris Medical School, and Léon Foucault, a physicist at the Paris Observatory. Donné had engravings made from the daguerreotypes and published them in 1845. A few years later in 1860, a photograph of the larynx was made by Czermak and in 1865 of the fundus of the eye, but it is Duchenne's publication in 1862 (in Boulogne) of the first book illustrated by clinical photographs that marks the entrance of photography into medical practice and clinical research.

In dermatology, the first photographs were published in the 1860s. In 1865, in London, Alexander John Balmanno Squire (1836–1908), an English surgeon, published the first atlas in dermato-venerology containing 12 photographs on albuminized paper colored by hand. In Paris, a few photographs of skin diseases were made at the Medical School, but it is at Saint-Louis Hospital that the first dermatological photographs were published by Hardy and Montméja in 1868, brought together in a book entitled *Photographic Clinic of the Saint-Louis Hospital*.

Hardy (1811–1893) had been the chief resident at Saint-Louis since 1851 and was appointed President of the First International Congress in Dermatology in Paris in 1889. Montméja was a former intern in ophthalmology, and when his first photographs were published, he had not yet finished his medical studies. From 1869 to 1873, he published in the *Revue photographique des hôpitaux de Paris* and succeeded in convincing the director of public assistance to set up the first photographic workshop in the Paris hospitals and (. . . .) "during the summer of 1866, Mr. Hardy learned about photographic experiments being carried out in England and confided to me (Montjéma) that he planned to study this new iconographic process in dermatology with him from then on (…). The colors entrusted to skillful hands are executed entirely before my eyes, with Mr. Hardy's final approval." The book contains 50 images classed to bring dermatology "into pathology and destroy these ideas of speciality that are founded on nothing true nor useful." Syphilis naturally occupies an important place (16 photos) and is added to cutaneous mycoses, scabies, impetigo, eczema, and alopecia.

Montméja colors the images by hand. His loyalty to the true morphology of the structures may be altered at times and, for certain images, he falls halfway between lithography and photography. With no particular lighting effects, the images uniformly present diseases and the diseased, from the disadvantaged of the social classes, who for the first time experience photography and seem frozen in a dramatic, sometimes almost theatrical, attitude, accentuated by the superimposition of the colors.

Fig. 40.16. Colored photograph of Impetigo (1868). Impetigo is a bacterial skin infection causes by a staphylococcus or streptococcus and characterized by blisters and pruritis. Gérard Tilles, Société française d'histoire de la dermatologie, Musée de l'hôpital Saint-Louis, Paris. Paper presented at the Symposium Jean-Louis Alibert, Barcelona, January 31, 1998, Nouv Dermatol 1998;**17**:271–275 (extract).

40.1.6.1 Photographers confronting disease *Sebastiao Salgado* was born in 1944 in Aimores, Brazil. After studying economics in Brazil and the United States, in 1971 Salgado finished his PhD at the University of Paris. During a trip to Africa in 1973, he borrowed his wife Lelia's camera and decided to change careers. Today he is one of the most respected photographers in photojournalism. Mr. Salgado works only in black and white, respects his subjects, and insists that what is happening to them has a broader meaning. This explains the force of a photographic work that bears witness to the fundamental dignity of the human being while forcefully rising against the violation of this dignity by war, poverty, and other injustices. Mr. Salgado has generously collaborated with various humanitarian organizations (e.g., UNICEF, United Nations High Commissioner for Refugees (UNHCR), the World Health Organization (WHO), Doctors Without Borders, Amnesty International). In September 2000 at the UN headquarters, he exhibited ninety portraits of displaced children extracted from his book *The Children: Refugees and Migrants*, a homage to 30 million people, for the most part homeless women and children. Mr. Salgado has donated the royalties that he receives for several of his photographs to the Global Movement for Children. He is currently illustrating the world campaign to eradicate poliomyelitis, a joint UNICEF and WHO project.

Kristen Ashburn, a young American photographer, has been working for the last 4 years on the ravages of AIDS in Zimbabwe. She has chosen to take a position as near as she can to the "human," to show more intimately the inhumane of the pandemic. Since 2001, Kristen Ashburn, 32, has made four documentaries in Zimbabwe on the havoc wreaked by AIDS. She prefers portraits, many taken indoors, as if to better grasp the intimacy of this frightening disease. In her black-and-white photos, she captures beings between life and death. Kristen's work shows just how much the pandemic has torn the traditional structures of this country to pieces, with a new generation gap, role reversal, and so on. Society is turned upside down. Zimbabwe already has more than 80,000 orphans, 35% of its population is HIV-positive, and one child dies of AIDS or opportunistic infections every 15 min. Some 2500 deaths a week are reported for the same reasons. Ashburn photographs Chitungwiza gravediggers preparing eight graves at once in a cemetery that is already saturated. In another photo, a group of people is attending a burial. But the legends clarifies: "Employers are beginning to limit the number of funerals their employees can attend because the absences are reducing their productivity." Ashburn also explains how religion has become the only medicine, because there are no medications, no money to pay for them, no public assistance, no basic necessities such as flour and sugar. Thus, 90% of Zimbabweans consult the services of traditional healers in this country where it is still said that AIDS is not the consequence of unprotected sexual relations, but it is sent by the ancestors who have not found rest.

Karen Kasmauki did not intend to become a professional photographer. She began by studying anthropology and religion at the University of Michigan. After receiving her degree, she received a grant to cover a study on oral histories in Tennessee, where she began to take photographs. Here she acquired a taste for photography and found a job as a photographer for the Virginia-Pilot in Norfolk. Five years later, she began freelancing as a photographer and proposed subjects to National Geographic, which allowed her to participate in a documentary on viruses that lasted more than 20 years. Karen Kasmauski has traveled to many countries covering the most devastating scourges of our time. The "Viruses: on the edge of life, on the edge of death" proposed by President J.F. Kennedy, was a turning point in her career. Whether it be her study on AIDS throughout the world, cholera in Bangladesh, or yellow fever in Brazil, Kasmauki has always followed her principle of photographing the ill as closely as possible, on very intimate terms. She thus became part of the most varied and unexpected worlds. She rubbed shoulders with prostitutes in Cambodia, drug addicts in the far reaches of Moscow, transvestites in Puerto Rico, and the bosses of a gay sauna in San Francisco. This long and gigantic study has earned her many articles in National Geographic, where she now works, and finally the publication of a book bringing together the major points in her prestigious journey: *From the Front Lines of Global Health*.

40.1.6.2 Photographers and disease Photography began to occupy an important place in art from the 1970s. Many photographers immortalized their suffering or that of their relations through the photographic medium, as self-portraits or portraits. Disease occupies an important place in this context, first and foremost AIDS, which at the beginnings of the disease, before triple therapy was accessible, degraded the body dramatically, arousing one of the greatest terrors of the twentieth century.

Fig. 40.17. "yeux" by Karen Kasmauski.

David Nebreda, a contemporary Spanish artist, remained shut away for more than 7 years to take pictures in which his body appeared mutilated, lacerated, or covered in excrement. (A book collects these photographs, published in 2000 by Leo Scheer.) The photographer wants to "incarnate the essential mythical figures of Western culture," in his own words. Nebreda's self-portraits, although very nearly unbearable, are highly esthetic. They are inspired by the classical themes of art, particularly Spanish baroque. It is this identity, inherited from the golden hints of conquest and the inquisitional torments, from the luxury of baroque renaissance and the torturing asceticism of the Counter Reformation, that Nebreda's art communicates. How not to recognize the mark of El Greco and Francisco Pacheco, and also Géricault whose *The Madwoman* appeared in his self-portraits like the quotation of a mother, Velázquez mirrored in *Parable of Mother and Son*, Goya, of course, the processions of flagellants and the terror of the *saëtas de patro soto*? Nebreda transcends his disease and physical decay by immortalizing himself in his paintings. It is a veritable work of exorcism that he took upon himself.

Robert Mapplethorpe, an American photographer, gathered inspiration from boxes of photos, found in New York homosexual nightclubs, staging men who were chained together or flogging themselves. A great admirer of Andy Warhol, Mapplethorpe developed in the underground artistic ferment of his time, photographing those around him. Among these subjects were porn stars, artists, composers, musicians, singers, the basis of the milieux, the hardest hit by the emerging AIDS. Mapplethorpe's first photos were considered shocking: He took what was going on around him quite instinctively, producing a portrait with no pretenses of this generation of young creators who roused a great deal of talk about them and who participated in removing taboos from AIDS and homosexuality. Little by little, Mapplethorpe's work became more refined: he preferred high-quality classical portraits, staging male and female nudes, and the personalities of his time. He also produced floral compositions and took up the subject of vanity in his last photographs. In 1987, he created the Robert Mapplethorpe Foundation whose goal was to promote photography and support medical research on AIDS.

Hervé Guibert, who was first and foremost a writer, also turned out an intimist photographic production around his personal experience. Homosexual, afflicted with AIDS, ending in suicide at the age of 36, Hervé Guibert placed the disease at the heart of his literary and photographic work. He provides several portraits of his companions, as well as a few self-portraits, showing in a very minimalist manner the exclusion and interior torments of the disease. Out of his mortal pain he made the great documentary of his life. He photographed himself for the newspapers, recounted his life in novel after novel, filmed himself dying for television. For Guibert, AIDS was the spectacular manifestation of his desire to die. Most certainly, AIDS was going to kill him. To defy it, he preferred to commit suicide, thus preventing it from deciding in his place.

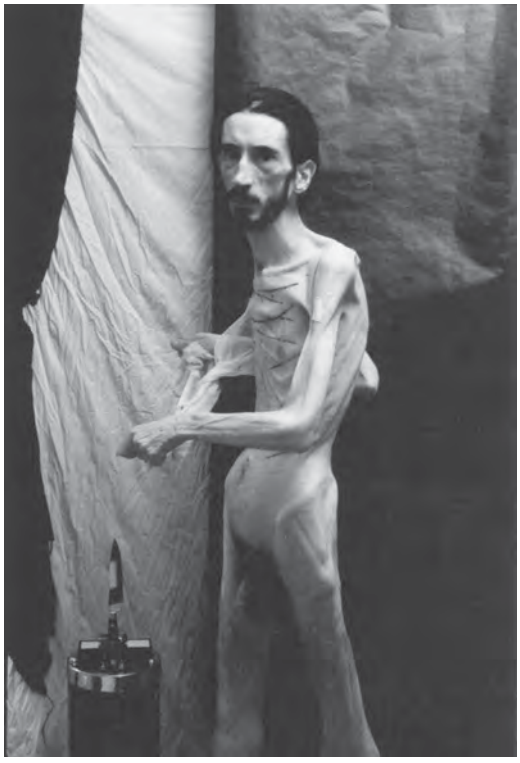

Fig. 40.18. Nude and Mother gift. David Nebrada 1989 "My mother gift: The knife with my name on it." David Nebrada. Color picture on argenetic paper, 102 × 75 cm Paris, galerie Leo Scheer. See color plates.

40.1.7 Graphic Arts

40.1.7.1 Drawings and water colors of medicinal plants Therapeutic medicinal plants were widely used against infectious diseases. They occupy a vital place in the traditional pharmacopeia and are imperative to modern research, along with other natural substances of medical interest, belonging as much to the plant kingdom as the animal kingdom, found on land or in the sea.

Although the use of herbariums was indispensable when it came to identifying and classifying plants, beyond the treatises in botany drawings were widely used for their descriptions. Plates most often showed the plants' morphology accurately and precisely. They were widely used, as much for their scientific value as for the artistic quality. They were exhibited in books, museums, and even in salons.

In medicine, this knowledge had to be transmitted to practitioners. The drawing of medicinal plants, along with coloring, illustrated medical treatises and botanical handbooks dedicated to medicine and the curative value of plants.

Only a few examples are presented here, chosen from illustrations of medicinal plants that act on bacteria and infectious syndromes: we are thinking of the original pharmacopeia of the School of Madagascar [8]. If generally speaking medicinal plants have varied actions on illness, the preparation and the dose are at the heart of the therapeutic activities, most often treating more the symptom than a targeted

Fig. 40.19. *Salvia coccinea* Jusieu *ex* Murray (Lamiaceae family) from the Medical Plants of Madagascar [8], the Tropical Sage or Scarlet Sage (Mint Family) present several medical properties including a strong anti-pyretic effect using a concoction of leaves and also a known and well used all over the Indian Ocean antibiotic outcome contained in the whole plant extract used to threat upper respiratory infections.

etiology. In their effects against infectious diseases, plants are recognized as having an antiseptic, antibiotic, anticatarrhal, or nematicide role, to go back to a term from Western medicine.

40.2 LITERATURE

Literature includes both the novel and poetry, as well as all ways of transcribing languages with the wide variety of handwritings, signs, ideograms, calligraphies, and so on.

Yet rock paintings, the art of modern humans, *Homo sapiens sapiens*, show no sign of infectious diseases. In the oldest wall art known, found in the "Chauvet caves" (Ardèche, France) and dating from 32,000 years ago, although certain images bring to mind healing rituals, nothing remotely interpretable as a representation involving infectious disease can be observed. The same is true for more recent Neolithic prehistory and the first permanent settlements with agriculture or animal husbandry, and in the first urban civilizations, from Mesopotamia to the Pharaonic civilization: traces of our topic cannot be found, and, other than a few limited examples of sculptures or bas-reliefs, infectious diseases are not in the picture at that time. However, in classical Antiquity, the first manuscripts considered in the West to be great literary works such as the Bible and the Homeric poems trace events believed to be manifestations of infectious diseases, which are either raised allegorically or described precisely.

We have therefore selected among the masterpieces of Western literature those works that make exemplary use of infectious disease as the object or subject of the novel, as actors or settings of a play. Several diseases, limited in number, have often inspired the writer because of their severity or their potential to affect the rich and the poor alike. This was most notably the case of the black plague, assimilated with death itself and magnified in all representations, literary or plastic, from the death dance to other spectacular manifestations such as the parades of the Flagellants and later in carnivals. The white plague (tuberculosis) was also a great theme, in a more recent time, used in particular by the Romantics for the slow and insidious suffering that it inflicted, leading inexorably to a dramatic and fatal end.

40.2.1 The Bible

The Bible, a primary and sacred text if ever there were one, unequivocally relates episodes of infectious epidemics. The oldest episodes first became a tradition transmitted orally and then were transcribed in manuscript form.

The first passages relating to infectious diseases are found in Exodus**,** which relates the episodes of the Hebrew people's departure from Egypt, events that date from the fifteenth century B.C. for the analysts to the thirteenth century B.C. for others. In the verses recounting the affair of the Ten Plagues of Egypt, for the first time the Old Testament tells of diseases of infectious character and epidemics, even if they are reported metaphorically, even esoterically. The episode ends thus: the Pharaoh refusing to allow the Hebrews to depart, Moses and his brother Aaron ask their god Yahweh several times to send down lightning on the Egyptians to force them to give up.

Several of the "plagues" described illustrate clearly an infectious character:

> (Exod.7.20–21) (. . .) And he lifted up the rod, and smote the waters that were in the river, in the sight of Pharaoh, and in the sight of his servants; and all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river; and there was blood throughout all the land of Egypt.

The river's waters were poisoned either with an infectious agent or with a toxic agent (chemical or biological).

> (Exod. 8.21) Else, if thou wilt not let my people go, behold, I will send swarms of flies upon thee, and upon thy servants, and upon thy people, and into thy houses: and the houses of the Egyptians shall be full of swarms of flies, and also the ground whereon they are.

In all likelihood, the flies are considered here as purveyors (vectors) of some plague for man and beasts.

(Exod. 9.2–3) For if thou refuse to let them go, and wilt hold them still, behold, the hand of the Lord is upon thy cattle which is in the field, upon the horses, upon the asses, upon the camels, upon the oxen, and upon the sheep: there shall be a very grievous murrain.

The term "murrain" (a pestilence or plague), nonspecific but infectious, is clearly expressed, striking in particular domestic ungulate animals.

(Exod. 9.10) And they took ashes of the furnace, and stood before Pharaoh; and Moses sprinkled it up toward heaven; and it became a boil breaking forth with blains upon man, and upon beast.

Here again this brings to mind the infection of scourges and the development of abscesses by bacterial or fungal agents, as well as leprosy, in the generic sense, at that time meaning a skin disease.

Further in the Bible, the two books of Samuel speak of similar events with the Hebrews settled in the Promised Land, punished by Yahweh:

(1 Sam. 5.9) (…) And it was so, that, after they had carried it about, the hand of the Lord was against the city with a very great destruction: and he smote the men of the city, both small and great, and they had emerods in their secret parts.

(2 Sam. 24.15) So the Lord sent a pestilence upon Israel from the morning even to the time appointed: and there died of the people from Dan even to Beersheba seventy thousand men. (…)

The Book of Chronicles relates even more precisely a probable acute intestinal infection.

(2 Chron. 21.14, 15) (…) behold, with a great plague will the Lord smite thy people, and thy children, and thy wives, and all thy goods: and thou shalt have great sickness by disease of thy bowels, until thy bowels fall out by reason of the sickness day by day.

In the Book of Ezekiel, it is remarkable to note how, for the first time, the people related the drama of infectious disease, the violence of war, and the misery of famine.

(Ezek. 6.11, 12) Thus saith the Lord God; Smite with thine hand, and stamp with thy foot, and say, Alas for all the evil abominations of the house of Israel! for they shall fall by the sword, by the famine, and by the pestilence. He that is far off shall die of the pestilence; and he that is near shall fall by the sword; and he that remaineth and is besieged shall die by the famine: thus will I accomplish my fury upon them.

This tragic trilogy is found again and again throughout time in a great number of accounts of epidemics, thereby becoming a strong element in the history of humanity, marked by the same spirit in art and politics to this very day.

Finally, in Revelations, the last book of the New Testament, the writer uses the same dramatic content:

Rev. 6.8. And power was given unto them over the fourth part of the earth, to kill with sword, and with hunger, and with death, and with the beasts of the earth.

We should also cite:

(Rev. 16.1) And I heard a great voice out of the temple saying to the seven angels, Go your ways, and pour out the vials of the wrath of God upon the earth.

(Rev. 16.2) And the first went, and poured out his vial upon the earth; and there fell a noisome and grievous sore upon the men which had the mark of the beast, and upon them which worshipped his image.

(Rev. 18.8) (…) Therefore shall her plagues come in one day, death, and mourning, and famine; and she shall be utterly burned with fire: for strong is the Lord God who judgeth her.

We can see, therefore, in this founding text that is the Bible, that calamities of all sorts, including infectious ones, are thrown at men by their God as a punishment for their sins. This interpretation—sin, divine vengeance—will be repeated, almost as a litany, throughout history, even today.

40.2.2 Homer: The Iliad (Song I) [58]

In the Iliad, the first part of the long Homeric poem, certainly sung at first according to oral tradition and later transcribed, from the beginning of the work we find in the vengeance of Apollo the scourge of a plague sent down by the lines of his arch.

(. . .) What god drove them to fight with such a fury? Apollo, the son of Zeus and Leto. Incensed at the king to he swept a fatal plague through the army—men were dying and all because Agamemnon spurned Apollo's priest.

(. . .) His prayer went up and Phoebus Apollo heard him. Down he strode from Olympus' peaks, storming at heart with his bow and hooded quiver slung across his shoulders. The arrows clanged at his hack as the geld quaked with rage, the god himself on the march and down he came like night. Over against the ships he dropped to a knee, let fly a shaft and a terrifying clash rang out from the great silver bow. First he went for the mules and circling dogs but then, launching a piercing shaft at the men themselves, he cut them down in droves—and the corps-fires burned on, night and day, no end in sight.

(. . .) Achille. So home we sail. . .if we can escape our death—if war and plague are joining forces now to crush the Argives.

In this Homeric episode, the war joins a "plague" to punish and spread hardship on men. Let us note the identification of arrows as purveyors of the pestilence. This will often appear later in images and text, in particular those of a religious nature.

40.2.3 The Black Death

The Black Death is an infectious disease caused by the bacillus *Yersinia pestis*, which naturally infects wild rodents as well as brown and black domestic rats. The bacillus is transmitted to humans when they are bitten by infected fleas that have fed on infected rats. Humans develop bubonic plague or pneumonic plague, and the illness can be secondarily transmitted either by infected fleas or directly from human to human through infected droplets [11].

40.2.3.1 Thucydides, the Peloponnesian War [95]

The Peloponnesian War opposed two Greek coalitions from 431 to 404 B.C., one led by Athens and the other by Sparta. Thucydides, the Athenian, was both an actor and a general, an observer and an exile, before becoming the brilliant conqueror, recognized as the first historian man of letters. He interests us here because in just a few pages his text describes the terrible epidemic that ravaged his city and region from March 430 to February 429. This is the first precise and detailed account of such an event. Since that time, historians and biologists have long wondered about the etiology of this epidemic, which is now commonly called the Plague of Athens.

Roussel, the translator of the French edition, places a long note at the beginning of Thucydides's pages on the plague: "Despite the precision of the details given by Thucydides, the doctors who have studied his text have not been able to come to an agreement on the name of the disease. One thing is certain: the 'plague' of Athens has nothing to do with the true plague. As for the rest, hypotheses will have to do. Some support a smallpox epidemic descending on a population affected with latent ergotism, 'poisoning caused by rye ergot fungus'; others favor the idea of measles that was exceptionally deadly because it sprang up in a population that had never known the disease; typhus could also be blamed. It must not be forgotten that certain diseases may have disappeared since the fifth century or that their clinical manifestations may have changed" [54].

(p. 114) (47) [These numbers correspond to section numbers of chatper II, number II of the book]

(. . .) the Peloponnesians and their allies invaded Attica (. . .). Not many days after their arrival in Attica the plague first began to show itself among the Athenians.(. . .) Neither were the physicians at first of any service, ignorant as they were of the proper way to treat it (. . .), nor did any human art succeed any better.

It should be noted, in just a few words, the narrator presents the emergence of this disease in an area and in a population that had not been affected before. This is confirmed later and shows the helplessness of those faced with this hitherto unheard-of scourge.

(p. 115–116) [49] That year then is admitted to have been otherwise unprecedentedly free from sickness; and such few cases as occurred, all determined in this. As a rule, however, there was no ostensible cause; but people in good health were all of a sudden attacked by violent heats in the head, and redness and inflammation in the eyes, the inward parts, such as the throat or tongue, becoming bloody and emitting an unnatural and fetid breath. These symptoms were followed by sneezing and hoarseness, after which the pain soon reached the chest, and produced a hard cough. When it fixed in the stomach, it upset it; and discharges of bile of every kind named by physicians ensued, accompanied by very great distress. In most cases also an ineffectual retching followed, producing violent spasms, which in some cases ceased soon after, in others much later. Externally the body was not very hot to the touch, nor pale in its appearance, but reddish, livid, and breaking out into small pustules and ulcers. But internally it burned so that the patient could not bear to have on him clothing or linen even of the very lightest description, or indeed to be otherwise than stark naked. What they would have liked best would have been to throw themselves into cold water, as indeed was done (. . .) The body meanwhile did not waste away so long as the distemper was at its height, but held out to a marvel against its ravages; thus when they succumbed, as in most cases, on the seventh or eighth day to the internal inflammation, they had still some strength in them. But if they passed this stage, and the disease descended further into the bowels, inducing a violent ulceration there accompanied by severe diarrhoea, this brought on a weakness which was generally fatal.

For the disorder first settled in the head, ran its course from thence through the whole of the body, and even where it did not prove mortal, it still left its mark on the extremities; for it settled in the privy parts, the fingers and the toes, and many escaped with the loss of these, some too with that of their eyes. Others again were seized with an entire loss of memory on their first recovery, and did not know either themselves or their friends.

The description given here of the symptoms of the disease and its progression is admirably precise, detailed, and full of imagery. Note that there is little sympathy in the tale of this plague; we are given a rather cold but rich clinical picture, despite a glimpse of emotion that shows through from time to time.

(p. 116) [50] But while the nature of the distemper was such as to baffle all description, and its attacks almost too grievous for human nature to endure, it was still in the following circumstance that its difference from all ordinary disorders was most clearly shown. All the birds and beasts that prey upon human bodies either abstained from touching them (though there were many lying unburied), or died after tasting them. In proof of this, it was noticed that birds of this kind actually disappeared; they were not about the bodies, or indeed to be seen at all. But of course the effects which I have mentioned could best be studied in a domestic animal like the dog.

At this stage of the description, the infectious aspect no longer leaves a doubt, and Thucydides speaks more of the ecology of the disease and advances a hypothesis on the mechanism of transmission.

(p. 117) [51] (. . .) By far the most terrible feature in the malady was the dejection which ensued when anyone felt himself sickening (. . .)

Yet it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience and had now no fear for themselves; for the same man was never attacked twice—never at least fatally.

(p. 118) [52] . . .for as the disaster passed all bounds, men, not knowing what was to become of them, became utterly careless of everything, whether sacred or profane. All the burial rites before in use were entirely upset. (. . .)

[53] Men now coolly ventured on what they had formerly done in a corner and not just as they pleased seeing the rapid transitions produced by persons in prosperity suddenly dying

> and those who before had nothing succeeding to their property. So they resolved to spend quickly and enjoy themselves, regarding their lives and riches as alike things of a day.

The description made of the moral atmosphere roughly resembles what will again be presented in the major writings on the plague and cholera epidemics during the Christian era, which the French translator, Denis Roussel, specified in a note: "Lucretius took up in his poem (*On the Nature of Things*, VI, 1038, 1286) this famous description of the plague of Athens. Boccaccio, describing the Florence plague, Defoe describing London's and Manzoni Milan's, each rivaled the Athenian historian."

40.2.3.2 Boccaccio, the Decameron [7] Another major narrator, Boccaccio (1313–1375), the first great Italian writer of prose, witnessed the ravages of the Great Plague of Florence, which he survived. He reports his experience of the epidemic in the preface and introduction of his most famous novel—a collection of short stories, *The Decameron*—where the plague is used as an argument for the narration: The meeting of seven young women and three young men who flee the city to find refuge in the "pure" air of the domain that he possesses in the country. To pass the time, they decide that every day for ten days each one will tell the others a story of an event they have witnessed or know to be true, totaling a collection of 100 short stories. The rules of the game stipulate that they cannot tell of the misfortunes of the day, in other words, the plague. The tales are gay, spiritual, even licentious, the novel situated in an erotic atmosphere, which provides the author with the pretext to satirize and criticize the society he lives in.

Let us cite a few extracts from the Introduction, the "beginning of the first day of the Decameron."

> p. 1–2 (. . .) for so is the painful recapitulation of the recent deadly plague, which occasioned hardship and grief to everyone who witnessed it or had some experience of it, and which marks the introduction of my work. (. . .) And just as sorrow will come to displace the most abundant happiness, so will the arrival of joy put paid to sorrow. (. . .) The era of the fruitful Incarnation of the Son of God had arrived at the year 1348 when the deadly plague reached the noble city of Florence, of all Italian cities the most excellent. Whether it was owing to the action of the heavenly bodies or whether, because of our iniquities, it was visited upon us mortals for our correction by the righteous anger of God, this pestilence, which had started some years earlier in the Orient, where it had robbed countless people of their lives, moved without pause from one region to the next until it spread tragically into the West. It was proof against all human providence and remedies (. . .)

Here we see that divine punishment is also brought to the forefront.

Further along, the author gives a number of precisions: for example, on buboes and their consequences; that people die within 3 days; that the infection is transmitted not only by the ill but also by objects belonging to them (p. 9: "a phenomenon much more surprising but observed, oh so many times"). There were those who were wise and those who were fearful, who fled the company of others or hid. However

Not only did the healthy incur the disease and with it the prevailing mortality by talking to or keeping company with the sick—they had only to touch the clothing or anything else that had come into contact with or been used by the sick and the plague evidently was passed to the one who handled those things).

> (p. 4) Others found the contrary view more enticing, that the surest remedy to a disease of this order was to drink their fill, have a good time, sing to their hearts' content, live it up, give free rein to their appetites-and make light of all that was going on. (. . .) This was easy enough to do because everyone had let his property go, just as he had let himself go, as if there was to be no tomorrow. (. . .) with our city in such a sorry state, the laws of God and men had lost their authority and fallen into disrespect in the absence of magistrates to see them enforced, for they, like everyone else, had either succumbed to the plague and lay sick, or else had been deprived of their minions to the point where they were powerless. This left everyone free to do precisely as he pleased.
> (. . .)
>
> (p. 4–5) (. . .) any number of men and women deserted their city and with it their homes and neighbourhoods, their families (. . .) as though the wrath of God, in visiting the plague on men to punish their iniquity, was never going to reach out to where they were; as though it was meant to harry only those remaining within their city walls, as though not a soul was destined to remain alive in the city, as though its last hour had come.
>
> Men and women alike were possessed by such a visceral terror of this scourge that a man would desert his own brother, uncle would forsake his nephew, sister her brother, and often a wife her husband. What is more, believe it or not, mothers and fathers would avoid visiting and tending their children, they would virtually disown them.

The abandonment of children by their parents struck a number of authors who related it in their works on a background of epidemic.

> (p. 6) Now this desertion of the sick by their neighbours, their families and friends, and the scarcity of servants, led to a practice hitherto unheard of: when a woman fell ill, she could be the neatest, prettiest, most refined of ladies, but she made no bones about being attended by a male, never mind his age, and displaying to him any part of her anatomy quite without embarrassment, just as would do with another of her sex, if her invalid condition required it. Conceivably this might have occasioned a certain lapse from the path of virtue among those women who subsequently recovered their health. (. . .) Not merely did many people die bereft of their attendant feminine company, all too many passed away without so much as a single witness. Barely a handful were accorded the benefit of seeing their dear ones in floods of compassionate tears: far from it, the new order called for quips and jollity more suited to a festive gathering. The womenfolk had largely suppressed their natural pity and become well practised this new frivolity to assure their own survival (. . .).

Boccaccio enjoys underscoring the fall of refined morals and social values in a time of epidemic, spreading death like a line of gunpowder spreads fire, striking down one and all. Later, carnivals ritualized the theme. The experience of the Great Plague and its permanent threat later developed a sort of frenzy for life that was taken up particularly by the dramatic arts.

> (p. 7) If you examined the situation of the common people, and even that of much of the middle class, it looked a great deal bleaker still: (. . .) Many there were who passed away in the street, by day as by night, while scores of those who died indoors only made their neighbours aware of their decease by the stench of their decaying corpses.

The power of words and the formation of sentences make the art of the perfect novelist that Boccaccio was; his great talent seems magnified by the reality of the epidemic. Before ending his relation to the plague, he devotes a page to the countryside, where the peasants showed themselves to be buried in the fear and fright of death, just as the city-dwellers were

> p. 8. As a result they, like the townsfolk, became feckless in their habits, neglecting their affairs and their possessions; indeed, far from encouraging their animals, their fields, their earlier labours to bear fruit, they all bent their best efforts to dissipating whatever came to hand.(. . .)
>
> Leaving the countryside and returning to the city, what more is there to say but that, what with the inordinate wrath of Heaven and doubtless also to some extent the cruelty of men, between March and July more than a hundred thousand human beings are in all certainty believed to have lost their lives within the walls of Florence: this as a result partly of the sheer inexorability of the plague, partly of the terror possessing the survivors, which prevented them from attending and ministering to in their need? (. . .) How many gallant men, how many fair women and bright young people whom anybody would have pronounced among the fittest—even physicians as eminent as Galen, Hippocrates, and Aesculapius would have sat down to breakfast with their families and friends only to find themselves dining that night with their forbears in the next world!

Boccaccio said no more. Having forcefully expressed his observations on the Great Plague and its horrors, he comes down to his subject: the reunion of the characters who push away the fear and perhaps death in telling more pleasant stories, cheerfully and often on a droll tone.

40.2.3.3 A journal of the plague year [28] It was impossible to ignore this book, written and published in 1722 by Daniel Defoe (1660–1731), the famous author of *Robinson Crusoe* and others: *Moll Flanders* and *Lady Roxana*. His literary vocation seems to have come rather late in life, trying his hand at poetry and pamphlet writing after the age of 40, then journalism, creating his own magazine, before launching into literature with *Robinson Crusoe* on the threshold of old age at nearly 60. But throughout his adult life he was a politician, often betting on the wrong horse, businessman and merchant with more experience in failure than in success, to the point of being put in the stocks in 1703. Impecunious and unlucky, he was often pursued for his debts, as happened again toward the end of his life. His need to rebuild himself was certainly one of the reasons that he started writing and publishing—to the great fortune of his readers and world literature, with a few masterpieces, then a dozen works that are still read with pleasure.

A Journal of the Plague Year, published in 1722, the same year as three hitherto unpublished novels, is a special piece of work. It should be remembered that this is fiction, a story and report more than a journal; some critics consider it a novel. It is not a personal account, or very little so: Defoe was 5 years old during the London plague of 1665, an age where the mind of a child is imbued for life by the exceptional and dramatic events that he may have lived. So why did he recount this epidemic, more than half a century after the facts? The French scholar today, Professor Henri-Hubert Mollaret, of the Institut Pasteur in Paris, specialist of the plague and its history, advances the idea that Defoe's purpose was to warn his contemporaries about the return of the plague, the most recent (and the last in Europe, but that was impossible to know at the time), having been the Marseille plague in 1720. Defoe may have wanted to warn of the horrors of the plague, instruct and tell how to best behave during a plague, underline the good and bad measures to take, and therefore taking as an example the 1665 plague in London as warning and advice.

His documentation is abundant and serious: he consulted archives, reported eyewitness accounts, and took down many anecdotes, without fabricating any it would seem. The shopkeeper's spirit of the narrator, and undoubtedly of Defoe himself, made him keep veritable accounts of the victims and costs, parish by parish. He highlighted the progress of the plague through these accounts: It blazed up in such and such a sector, calmed down or went out there, and resurfaced elsewhere, a morbid cartographic and topographic description of the city.

Finally, this account is also interesting for its enumeration and description of the exceptional measures taken by the authorities and public services in their attempt to limit the epidemic, measures that had a certain success, notably confinement, cruel but effective: Every house that had a diseased person was closed up with its occupants, guarded by watchmen. Another measure limited the movement of the healthy. In the seventeenth century, Europe organized the fight against an arising epidemic, measures that in 1720 would prevent the plague in Marseille from spreading too far, which would consequently go little beyond the limits of Provence and Languedoc, despite an even greater number of victims and the sacrifice of healthcare, government, and service personnel (this was the last great surge of the plague in Western Europe). Professor H.H. Mollaret, in the preface of the French edition, writes (p. 21) [28]

> Daniel Defoe has his narrator communicate in a style that was voluntarily cold, distant, rather pretentious, with no purple

passage, emotion rare despite the grandiloquence in the description of scenes where the actors sink into great fear. He transcribes the commercial spirit and mercantile morals of the century that would soon be called the Enlightenment. His narrator is a well-off saddler (note that the author's uncle was a saddle maker in London at the time of the plague, which he had witnessed; would he have kept a journal? Or his nephew, who had kept memories of the adults' hardships and misfortunes, had listened attentively, which reinforces the truthfulness of the work).

Because this merchant, although well-off, has neither the taste nor the habit of writing, Defoe gives the *Journal* a construction and a form that are voluntarily awkward, heavy, with unbearable repetitions that some editions believed had to be removed, regrettable because these repetitions, intentional on Defoe's part, contributed to defining his character and, most especially, because they hammered like a monotonous knell the progression of the epidemic (. . .)

A great speechifier with a limited vocabulary, the saddler, sententious, moralizing, and puritanical, could not keep his *Journal* without these continual repetitions. There is some of the Monsieur Prudhomme about him, and Monsieur Homais, du Bouvard, and Pécuchet, too: he is proud of his city, but disputes the measures of the town councilmen; he is proud of himself, because in the end he did not flee London, after many a prevarication it is true, and feels very personally protected by God (. . .)

This quite unattractive writing is also, paradoxically, the very charm and interest of this reference work *A Journal of the Plague Year*.

We have chosen the following quotations among the many others that are equally interesting:

p. 30 Death was before their Eyes, and every Body began to think of their Graves, not of Mirth and Diversions.

p. 38 Orders conceived and published by the Lord Mayor and Aldermen of the City of London, concerning the infection of the Plague, 1665.

Whereas in the Reign of our late Sovereign King James, of happy Memory, an Act was made for the charitable Relief and ordering of Persons infected with the Plague; whereby Authority was given to justices of the Peace, Mayors, Bayliffs and other head Officers, to appoint within their several Limits, Examiners, Searchers, Watchmen, Keepers, and Buriers for the Persons and Places infected, and to minister unto them Oaths for the Performance of their Offices. And the same Statute did also authorize the giving of other Directions, as unto them for the present Necessity should seem good in their Discretions. It is now upon special Consideration, thought very expedient for preventing and avoiding of Infection of Sickness (if it shall so please Almighty God) that these Officers following be appointed, and these Orders hereafter duly observed. (. . .)

p. 40. Sequestration of the Sick. As soon as any Man shall be found by this Examiner, Chirurgeon or Searcher to be sick of the Plague, he shall the same Night be sequestred in the same House, and in case he be so sequestred, then, though he afterwards die not, the House wherein he sickned, should be shut up for a Month, after the use of the due Preservatives taken by the rest.

p. 45 Plays. That all Plays, Bear-Baitings, Games, singing of Ballads, Buckler-play, or such like Causes of Assemblies of People, be utterly prohibited. (. . .)

Feasting Prohibited. That all publick Feasting, and particularly by the Companies of this City, and Dinners at Taverns, Alehouses, and other Places of common Entertainment be forborn till further Order and Allowance; and that the Money thereby spared, be preserved and employed for the Benefit and Relief of the Poor visited with the Infection.

The above quotations are only a few examples of the many administrative measures cited by Defoe, taken by the London authorities, the Lord Mayor and his two sheriffs.

p. 54 But I come back to the Case of Families infected, and shut up by the Magistrates; the Misery of those Families is not to be express'd, and it was generally in such Houses that we heard the most dismal Shrieks and Out-cries of the poor People terrified, and even frighted to Death, by the Sight of the Condition of their dearest Relations, and by the Terror of being imprisoned as they were.

p. 55 . . . but there were innumerable such like Cases; and it was seldom, that the Weekly Bill came in, but there were two or three put in freighted, that is, that may well he called, freighted to Death: But besides those, who were so freighted to die upon the Spot, there were great Numbers freighted to other Extremes, some freighted out of their Senses, some out of their Memory, and some out of their Understanding:

And then there were all those who masked their fear or defied the epidemic in debauchery:

p. 62 . . . but there was a dreadful Set of Fellows that used their House, and who in the middle of all this Horror met there every Night, behaved with all the Reveling and roaring extravagances.

p. 72 This put it out of Question to me, that the Calamity was spread by Infection, that is to say, by some certain Steams, or Fumes, which the Physicians call Effluvia, by Breath, or by the Sweat, or by the Stench of the Sores of the sick Persons, or some other way, perhaps, beyond even the Reach of the Physicians themselves, which Effluvia affected the Sound, who come within certain Distances of the Sick, immediately penetrating the Vital Parts of the said sound Persons, putting their Blood into an immediate ferment, and agitating their Spirits to that Degree which it was found they were agitated; and so those newly infected Persons communicated it in the same Manner to others; (. . .) likewise the Opinion of others, who talk of infection being carried on by the Air only, by carrying with it vast Numbers of Insects, and invisible Creatures, who enter into the Body with the Breath, or even at the Pores the Air, and there generate, or emit most accute Poisons, or poisonous Ovx, or Eggs, which mingle themselves with the Blood, and so infect the Body; a Discourse full of learned Simplicity, and manifested to be so by universal Experience (. . .)

p. 77–78 In these Walks I had many dismal Scenes before my Eyes, as particularly of Persons falling dead in the Streets, terrible Shrieks and Skreekings of Women, who in their Agonies would throw open their Chamber Windows, and cry out in a dismal Surprising Manner; it is impossible to describe the Variety of Postures, in which the Passions of the Poor People would Express themselves.

Passing thro' Token-House-Yard in Lothbury, of a sudden a Casement violently opened just over my Head, and a Woman gave three frightful Skreetches, and then cried, Oh! Death,

Death, Death! in a most inimitable Tone, and which struck me with Horror and a Chillness, in my very Blood.

p. 98–99 As the Desolation was greater, during those terrible Times, so the Amazement of the People increased; and a thousand unaccountable Things they would do in the violence of their Fright, as their Distemper, and this part was very affecting. (. . .) I suppose the World has heard of the famous Soloman Eagle an Enthusiast: He though not infected at all, but in his Head, went about denouncing of judgment upon the City in a frightful manner; sometimes quite naked, and with a Pan of burning Charcoal on his Head: What he said pretended, indeed I could not learn.

p. 116 Wherefore, were we ordered to kill all the Dogs and Cats: But because as they were domestic Animals, and are apt to run from House to House, and from Street to Street; so are capable of carrying the Effluvia or Infectious Steams of Bodies infected, even in their Furs and Hair; and therefore, it was that in the beginning of the Infection.

p. 146–147 Another thing might render the Country more strict with respect to the Citizens, and especially with respect to the Poor; and this was what I hinted at before, namely, that there was a seeming propensity, or a wicked Inclination in those that were Infected to infect others.

There have been great Debates among our Physicians, as to the Reason of this; some will have it to be in the Nature of the Disease, and that it impresses every one that is seized upon by it, with a kind of a Rage, and a hatred against their own Kind, as if there was a malignity, not only in the Distemper to communicate itself, but in the very Nature of Man, prompting him with evil Will, or an evil Eye, that as they say in the Case of a mad Dog, who tho' the gentlest before of any of his Kind, yet then will fly upon and bite any one that comes next him and those as soon as any, who had been most observed by him before.

Others placed it to the Account of the Corruption of humane Nature, which cannot bear to see itself more miserable than others of its own Specie, and has a kind of involuntary Wish, that all Men were as unhappy, or in as bad a Condition as itself.

p. 147 I choose to give this grave Debate a quite different turn, and answer it or resolve it all by saying, That I do not grant the Fact.

p. 152 . . . shutting up Houses . . . confined the distempered People, who would otherwise have been both very troublesome and very dangerous in their running about Streets with the Distemper upon them, which when they were delirious, they would have done in a most frightful manner; and as indeed they began to do at first very much, 'till they were thus restrained (. . .)

Here follows the account of a woman being attacked.

pp. 152–153 When she see he would overtake her, she turned, and gave him a Thrust so forcibly, he being but weak, and pushed him down backward: But very unhappily, she being so near, he caught hold of her, and pulled her down also; and getting up first, mastered her, and kissed her; and which was worst of all, when he had done, told her he had the Plague, and why should not she have it as well as he. She was frighten enough before, being also young with Child; but when she heard him say, he had the Plague, she screamed out and fell down in a Swoon, or in a Fit, which tho' she recovered a little, yet on her in a very few Days, and I never heard whether she had the Plague or no.

p. 225 (. . .) however the others aver the Truth of it, yet I rather chuse to keep to the public Account; seven and eight thousand per Week is enough to make good all that I have said of the Terror of those Times. (. . .)

And then the disease disappeared.

p. 230 However, in general, prudent cautious People did enter into some Measures for airing and sweetening their Houses, and burnt Perfumes, Incense, Benjamin, Rozin, and Sulphur in the Rooms close shut up, and then let the Air carry it all out with a Blast of Gun-powder; others caused large Fires to be made all Day and all Night, for several Days and Nights; by the same Token, that two or three were pleased to set their Houses on Fire, and so effectually sweetened them by burning them down to the Ground.

Typical English humor shows up under the serious countenance of the character, but the account is possible as the great epidemic comes to a close: in other words, better nothing than some new risk. In reference to the great 1666 fire of London

p. 230 (. . .) But the Time was not fully come, that the City was to be purged by Fire, nor was it far off; for within Nine Months more I saw it all lying in Ashes.

p.232 (. . .) The Circumstances of the Deliverance were indeed very remarkable, as I have in part mentioned already, and particularly the dreadful Condition, which we were all in, when we were, to the Surprise of the whole Town, made joyful with the Hope of a Stop of the Infection. Nothing, but the immediate Finger of God, nothing, but omnipotent Power could have done it; the Contagion despised all Medicine, Death rag'd in every Corner; and had it gone on as it did then, a few Weeks more would have clear'd the Town of all, and every thing that had a Soul. (. . .)

p.363 (. . .) In the Middle of their Distress, when the Condition of the City of London was so truly calamitous, just then it pleased God, as it were, by his immediate Hand to disarm this Enemy; the Poyson was taken out of the Sting, it was wonderful, even the Physicians themselves were surprised at it; wherever they visited, they found their Patients better, either they had sweated kindly, or the Tumours were broke, or the Carbuncles went down, and the Inflammations round them changed Color, or the Fever was gone, or the violent Headache was asswaged, or some good Symptom was in the Case; so that in a few Days, every Body was recovering (. . .)

p. 234. Nor was this by any new Medicine found out, or new Method of Cure discovered, or by any Experience in the Operation, which the Physicians or Surgeons had attain'd to; but it was evidently from the secret invisible Hand of him, that had at first sent this Disease as a judgment upon us; and the Atheistic part of Mankind call my Saying this what they please, it is no Enthusiasm; it was acknowledged at that time by all Mankind; the Disease was enervated, and its Malignity spent, and let it proceed from whatsoever it will, let the Philosophers search for Reasons in Nature to account for it by, and labour as much as they will to lessen the Debt they owe to their Maker; those Physicians, who had the least Share of Religion in them, were obliged to acknowledge that it was all supernatural, that it was extraordinary, and that no Account could be given of it.

> p.235 (. . .) I can go no farther here, I should be counted censorious, and perhaps unjust, if I should enter into the unpleasant Work of reflecting, whatever Cause there was for it, upon the Unthankfulness and Return of all manner of Wickedness among us, which I was so much an Eye-Witness of my self; (. . .)

Daniel Defoe thus ends his account, noting gravely and not very optimistically on the wisdom of men. Did he not have the experience of an entire life when he wrote this pseudo-journal? Nonetheless it is the same man, Defoe, who a few years earlier had created Robinson Crusoe, raised to the heights of myth, a character who represents one of the constants of humanity, as his author says:

> Invincible patience in the worse misfortune, the tireless and indomitable reason in the most discouraging circumstances.

Yesterday: From Dark Romanticism and the Gothic: Mary Shelley—and Byron—Edgar Allan Poe

Gothic enters literature with the fairy tales (Charles Pairault, end of the seventeenth century, the Grimm brothers a century later), tales that were long claimed to be written for children, many of them containing all the ingredients and recipes of nineteenth century Gothic literature [9], today replaced by science fiction, although this also appears early, as demonstrated by the author and book we wish to speak of here: Mary Shelley. Infectious, contagious, epidemic disease was sometimes used by writers of this literary genre born with Romanticism (a dark Romanticism), which has endured—it reinforces the harrowing atmosphere and setting of the story, isolating, confining, or destroying, its heroes; or the epidemic is used as metaphor.

Mary Shelley (1797–1851). Through her life and work, Mary Shelley was one of the most surprising and even most extraordinary women of literary history, of history, nothing more and nothing less. Her mother, Mary Godwin, née Wollstonecraft, died in a purpura fever after giving birth to her daughter. Essayist and pamphleteer, she was one of the first feminists, and in 1792 published, among other things: Demands for Women's Rights. Her father, William Godwin (1756–1836) was a very well-known publicist, novelist, and philosopher of his time. A former pastor, he turned atheist under the influence of the French philosophers, "he devoted himself to the study of social matters. Questioning liberalism and the right to property, he formulated the principles of a society without government in which every member would benefit from shared work in proportion to his needs" [32]. This brilliant, knowledgeable father, an unusual character, gave Mary a highly original education for a girl at this time, raising her somewhat like a boy and teaching her subjects that were far from the usual education of young ladies. Her parents, her inborn intelligence and vivaciousness of spirit, literature (Godwin also tended a bookshop), philosophy, and the sciences made Mary a gifted child and adolescent. William Godwin also had an influence on the two great poets of English Romanticism, becoming their friend and mentor: Perçy Bysshe Shelley (1792–1822) and George Gordon Noel Byron, Lord Byron (1788–1824). This trio of men was to be, in one fashion or another, the men of Mary's life, men who were admired, venerated, then rejected, even hated.

In 1814, Mary became Shelley's mistress; although he was married, he divorced for her 2 years later. This is the time when they made friends with Byron, a great admirer of Godwin. With Mary's half-sister, Claire Clairmont, a year younger, the couple left for Geneva in May 1816, where they met up with Byron, who put them up in a luxurious villa that he had rented. Byron, just divorced after a scandalous affair (he had had an incestuous relation with his own half-sister), who was incapable of getting to know a woman without falling in love with her and desiring her, seduced Claire, who became his mistress for the summer (she gave birth to his child after their separation).

On the evening of June 16, 1816, on a rainy, stormy night, Byron suggests to his friends (a Geneva doctor, one of his friends, is also present) that they each write "a Gothic ghost story" (a fashionable theme with the Romantics). Byron and Mary set to work, the others have left no trace. The poet wrote a novel entitled *The Vampire*, which would be finished by his secretary; this text inaugurated a genre and a myth: the myth of the "lords of the night," whose literary and film success has never ceased. For her part, Mary wrote the basic structure of a short story that she called "Frankenstein, or the Modern Prometheus." Thus, on the same night, the two greatest myths of Gothic literature were born in the imaginative spirit of two brilliant young writers, one a man and one a woman. Mary developed and finished the novel in the following months—she was 19 years old. She had to wait until 1818 for a London publisher to publish it. She was disparaged as much as she was congratulated by her peers (among whom Walter Scott), but the book enjoyed considerable success.

Let us simply summarize the story, less well-known to today's public: Victor Frankenstein, a young Geneva scholar, dreams of making a perfect human, one who is handsome, good, and moral, who is protected from disease and epidemics. To reach his goal, he steals bodies in a neighboring cemetery (some have died of infectious diseases, the only relation to our subject). He manages to put the pieces together and give life to the reconstituted being. But his Creature is physically and physiognomically a monster, a 7-foot-tall monster, with a horrible face. Frankenstein, shattered, abandons the fruit of his work. But his creature is alive, endowed with a mind, intelligence, and sensitivity. He asks only that his creator make him a companion in his image to live in this world that he does not understand. When Frankenstein refuses, the Creature takes vengeance, kills, massacres, notably the young scholar's family that he pursues throughout the world with his hatred. After a last battle near the North Pole, the creator dies and the Creature disappears.

Mary Shelley took inspiration from conversations with her husband, a lover of the physical and natural sciences; she also knew the history of the German alchemist Nippel, "a thief of cadavers to search for the principle of life." At this

time of passion for progress, the sciences, and technology, the young woman sensed the risks for humanity, for social and family life, of a science with no limits and with no conscience (Rabelais's famous "ruin of the soul"). She thought that scholars would become Prometheus, demiurges, who would end up believing they were God. The Creature is an unhappy living being seeking happiness, but who finds himself deprived of it. But he is no more than a parasite who can kill like an epidemic. This is how Frankenstein and Byron's imagined vampires relate to our subject.

Mary Shelley would soon suffer the misfortunes of life: the death of her young children, her great poet of a husband whom she adored, drowned in the Mediterranean, in the Gulf of Genoa in July 1822; and then, on April 16, 1824, her beloved great poet friend, Lord Byron, died in Greece. Contrary to what many believe, the foreign hero of Greek independence from the Turkish Empire, did not die on the battlefield, but of a rather mysterious fever (flue, typhus?) that took him in just a few days. Shelley, had tuberculosis. Mary wrote in her journal on May 15, 1824: "Why I am doomed to live on seeing all expire before me . . . at age 26 I am in the condition of an aged person, all me old friends are gone." The day before she had written: "The last man! Yes, I may well describe that solitary being's feelings, feeling myself as the last relic of a beloved race, my companions extinct before me." From these lamentations, this desolation, this solitude, this expression of despair, *The Last Man* was born, her second great novel, published in 1826. It was less famous than *Frankenstein*, but also important, at least for her—in her mind, the last man is herself!

The story takes place 2073: the kind of England has abdicated. Five characters: three men—Lionel, Adrian, and Lord Raymond—who will become the Lord Protector of England; two women—Idris, Adrian's sister who marries Lionel, Perdita, Lionel's sister who marries Lord Raymond. The five friends and relatives by marriage live a romantic life, travel, before Lord Raymond turns ambitious, and, with his repeated affairs and dreams of glory, shatters the harmony. This is the story of the Shelleys and the Byrons. Also represented, although ambiguously, is the father and guide, William Godwin. They will all die but one: Perdida kills herself, the others drown or die of the plague that ravages Europe and decimates humanity. Lionel Verney (Mary Shelley) is "the last man," more in a figurative sense than in the literal sense. Sumeeta Patnik [85], a current commentator, has written:

> The spread of the plague throughout Europe is Mary Shelley's assessment of the failure of Shelley's utopian ideals to support his wife and children. (. . .) She repudiates Adrian's fragile sensibility and decision to never wed and take responsibility for a family—feelings that she shared about her late husband. For Mary Shelley, the plague serves as a metaphor for the failure of the utopian ideal. In her assessment, by rejecting utopian ideals, her father, Percy and Lord Byron have rejected the traditional values that keep a society and a family together and for Mary Shelley, that was their greatest failure. The novel can be viewed as Mary Shelley's way of coming to terms with the deaths of Lord Byron and her husband as well the loss of the ideal that their lives represented. *The Last Man* is Mary Shelley's last Gothic novel and by 1829, which is considered the end of the Romantic period, the young widow has decided to move on.

Mary Shelley (*The Last Man* [76]):

> "Oh, that death and sickness were banished from our earthly home! That hatred, tyranny, and fear could no longer make their lair in the human heart . . . the choice is with us; let us will it, and our habitation becomes a paradise. For the will of man is omnipotent, blunting the arrows of death, soothing the bed of disease, and wiping away the tears of agony." (cited by Julie K. Schuetz).

Edgar Allan Poe (1809–1849) "The Masque of the Red Death" [86]

Born in Boston, the son of poor actors and an orphan at 3 years of age, Poe was adopted by a rich tobacco plantation owner in Richmond, Virginia, John Allan. He studied in England and Virginia, quarreled with his adoptive father, attempted a military career but was dismissed from West Point in 1830, by which time he had already begun publishing poems. He moved in with his aunt in Boston and in 1835 he married his 14-year-old cousin, Virginia. A literary critic, in 1838 he published *The Narrative of Arthur Gordon Pym of Nantucket*, which influenced Joseph Conrad and Jules Verne, among others. The next year, he published his first *Tales of Grotesque and Arabesque*. An alcoholic and possibly addicted to drugs, he continued to write and publish his poetry (particularly prose poems). His work did not sell well; he remained in poor, even in poverty. The death of the women he loved—Virginia in 1847—finished him. He was found dead one night in a stream in Baltimore. Long scorned in his country, his writing became a true cult in France, with Charles Baudelaire's wonderful translations. He influenced the history of French literature of the second half of the century. He remains the master of ratiocination and horror stories [33].

> "The Masque of Red Death" is one of his beautifully written pieces, a short story that recounts in just four pages the captivating story of infection and death that could have lasted two hundred pages.
>
> The "Red Death" had long devastated the country. No pestilence had ever been so fatal, or so hideous. Blood was its Avatar and its seal—the redness and horror of blood. There were sharp pains, and sudden dizziness, and then profuse bleeding at the pores, with dissolution. The scarlet stains upon the body and especially upon the face of the victim, were the pest ban which shut him out from the aid and from the sympathy of his fellow men. And the whole seizure, progress, and termination of the disease, were the incidents of half an hour.
>
> But the Prince Prospero was happy and dauntless and sagacious. When his dominions were half depopulated, he summoned to his presence a thousand hale and light-hearted friends from among the knights and dames of his court, and with these retired to the deep seclusion of one of his castellated abbeys. (. . .) The courtiers, having entered, brought furnaces and massy hammers and welded the bolts. They resolved to leave means

> neither of ingress nor egress to the sudden impulses of despair or of frenzy from within. (. . .) With such precautions the courtiers might bid defiance to contagion. The external world could take care of itself. In the meantime it was folly to grieve, or to think. The prince had provided all the appliances of pleasure. There were buffoons, there were improvisatory, there were ballet-dancers, there were musicians, there was Beauty, there was wine. All these and security were within. Without was the "Red Death."
>
> It was toward the close of the fifth or sixth month of his seclusion, and while the pestilence raged most furiously abroad, that the Prince Prospero entertained his thousand friends at a masked ball of the most unusual magnificence.
>
> It was a voluptuous scene that masquerade. (. . .)

The author describes the seven ballrooms, lined one after another, their great doors open, each decorated in a different color, lighted through stained glass windows of the same color, with windows giving only onto lateral corridors with a great fire burning in front of each one. The last room was dressed in black, but with the stained glass windows in red. In this room there was a gigantic ebony clock, striking at each hour, the sound was so "clear and loud and deep and exceedingly musical, but of so peculiar a note and emphasis that, at each lapse of an hour, the musicians of the orchestra were constrained to pause, momentarily, in their performance, to harken the sound; and thus the waltzers perforce ceased their evolutions; and there was a brief disconcert of the whole gay company; and, while the chimes of the clock yet rang, it was observed that the giddiest grew pale, and the more aged and sedate passed their hands over their brows as if in confused reverie or medication." (. . .)

> But, in spite of these things, it was a gay and magnificent revel. The tastes of the duke were peculiar. (...) His plans were bold and fiery, and his conceptions glowed with barbaric lustre. (. . .) There were arabesque figures with unsuited limbs and appointments. There were delirious fancies such as the madman fashions. There was much of the beautiful, much of the wanton, much of the bizarre, something of the terrible, and not a little of that which might have excited disgust. To and fro in the seven chambers there stalked, in fact, a multitude of dreams. And these—the dreams—writhed in and about, taking hue from the rooms, (. . .)
>
> (. . .) But to the chamber which lies most westwardly of the seven there are now none of the maskers who venture; for the night is waning away; and there flows a ruddier light through the blood-coloured panes; and the blackness of the sable drapery appalls (. . .)
>
> But these other apartments were densely crowded, and in them beat feverishly the heart of life. And the revel went whirlingly on, until at length there commenced the sounding of midnight upon the clock. (. . .) and thus it happened, perhaps, that before the last echoes of the last chime had utterly sunk into silence, there were many individuals in the crowd who had found leisure to become aware of the presence of a masked figure which had arrested the attention of no single individual before. And the rumor of this new presence having spread itself whisperingly around, there arose at length from the whole company a buzz, or murmur, expressive of disapprobation and surprise—then, finally, of terror, of horror, and of disgust.
>
> In an assembly of phantasms such as I have painted, it may well be supposed that no ordinary appearance could have excited such sensation. In truth the masquerade licence of the night was nearly unlimited; but the figure in question had out-Heroded Herod, and gone beyond the bounds of even the prince's indefinite decorum. There are chords in the hearts of even the most reckless which cannot be touched without emotion. Even with the utterly lost, to whom life and death are equally jests, there are matters of which no jest can be made. (. . .) The figure was tall and gaunt, and shrouded from head to foot in the habiliments of the grave. The mask which concealed the visage was made so nearly to resemble the countenance of a stiffened corpse that the closest scrutiny must have had difficulty in detecting the cheat. (. . .) But the mummer had gone so far as to assume the type of the Red Death. His vesture was dabbled in *blood*—and his broad brow, with all the features of the face, was besprinkled with the scarlet horror.
>
> When the eyes of the Prince Prospero fell upon this spectral image (which, with a slow and solemn movement, as if more fully to sustain its *rôle*, stalked to and fro among the waltzers) he was seen to be convulsed in the first moment with a strong shudder either of terror or distaste; but, in the next, his brow reddened with rage.

The prince gave the order to lay hold of the hideous individual. He stood with "a group of pale courtiers by his side." The mask advanced "with a deliberate and stately step," coming closer to the prince. Nobody dared to stop him. He goes from room to room, to return to the black and red room. Exasperated, he rushed after him, with a dagger in his hand. The strange and terrible individual turned toward him. "There was a sharp cry—and the dagger dropped gleaming upon the sable carpet, upon which, instantly afterward, fell prostrate in death the Prince Prospero."

> Then, summoning the wild courage of despair, a throng of the revelers at once threw themselves into the black apartment, and, seizing the mummer, whose tall figure stood erect and motionless within the shadow of the ebony clock, gasped in unutterable horror at finding the grave cerements and corpse-like mask, which they handled with so violent a rudeness, untenanted by any tangible form.
>
> And now was acknowledged the presence of the Red Death. He had come like a thief in the night. And one by one dropped the revellers in the blood-bedewed halls of their revel, and died each in the despairing posture of his fall. And the life of the ebony clock went out with that of the last of the gay. And the flames of the tripods expired. And Darkness and Decay and the Red Death held illimitable dominion over all.

40.2.3.4 From real to fiction: The Plague by Albert Camus [12] Because he wrote at the end of the Second World War and because he exalted human solidarity in the face of malevolence, this novel by Albert Camus was considered an allegory of the political violence that the world had just come through, one of total brutality and extreme cruelty,

through the fault of a totalitarian regime: the plague is Naziism, and the rats the symbol of human malevolence. The author refutes nothing. What interested him was the behavior of men in a context of terror, in this case, caused by nature. His last page shows that, for him, one must always remain hopeful, and "that there are more things to admire in men than to despise." But this optimist is misleading and, as the following commentary, The "Robert Surname Dictionnary" [33], says: "The tale symbolizes the absurdity of the human condition, against which there are only ephemeral victories," which is said loud and clear at the end of the book's last sentence: "the day would come when, for the bane and the enlightening of me n, it would rouse up its rats again and send them forth to die in a happy city."

The novel recounts an epidemic of the plague in Oran (where Camus was born), although imaginary, there had been a few contained epidemics of plague in Algeria during the 1930s and 1940s. The story is a third person account of the daily life of a doctor, Doctor Rieux, during the catastrophe, whose entire life and medical vocation must have prepared him for this, and who does his best to do his duty with humility. ("The thing was to do your job as it should be done.") This is a tale told in a neutral tone, at times minimalist, almost modest, with rare outbursts of lyricism. The narrator is revealed at the end of the book to be Doctor Rieux. Camus describes with great precision what could be the administrative and social situation of a population, confined to the town because of a modern epidemic.

We begin with Camus's sentence, which, after a few pages presenting the town, opens his tale with the discovery of dead rats.

> (p. 15) But the situation worsened in the following days. There were more and more dead vermin in the streets, and the collectors had bigger truckloads every morning. On the fourth day the rats began to come out and die in batches. From basements, cellars, and sewers they emerged in long wavering files into the light of day, swayed helplessly, then did a sort of pirouette and fell dead at the feet of the horrified onlookers.

Roughly 60 years ago, in Camus's time, science knew nearly everything there was to know about the plague and its cycle: lethal bacillus, the flea as reservoir that then bites and infects the rat and humans. However, the image of sick rats fleeing their subterranean gloom to come out and die in the open air, in the light, and in contact with humans remains just as powerful.

> (p. 17) M. Michel's eyes were fever-bright and he was breathing wheezily. The old man explained that, feeling "a bit off color," he had gone out to take the air. But he had started feeling pains in all sorts of places—in his neck, armpits, and groin—had been obliged to turn back and ask Father Paneloux to give him an arm.
>
> (p. 22) (. . .) Two hours later the doctor and Mme. Michel were in the ambulance bending over the sick man. Rambling words were issuing from the gaping mouth, thickly coated now with sordes. He kept on repeating: "Them rats! Them damned rats!" His face had gone livid, a grayish green, his lips were bloodless, his breath came in sudden gasps. His limbs spread out by the ganglia, embedded in the berth as if he were trying to bury himself in it or a voice from the depths of the earth were summoning him below, the unhappy man seemed to be stifling under some unseen pressure. His wife was sobbing.

As did other writers of the twentieth century who used an infectious disease in their writing, Camus did his research and precisely described the disease and its symptoms on patients and victims. But the writer's art reinforces the descriptions—the following quotation ends in a short, few word phrase that says it all: the patient is going to die and his wife, like the doctor, knows it.

> (p. 35–36) I was in China for a good part of my career, and I saw some cases in Paris twenty years ago. Only no one dared to call them by their name on that occasion. The usual taboo of the public mustn't be alarmed, that wouldn't do at all. And then, as one of my colleagues said, "It's unthinkable. Everyone knows it's ceased to appear in western Europe." Yes, everyone knew that—except the dead men. Come now, Rieux, you know as well as I do what it is.
>
> (p. 37) (. . .) Everyone knows that pestilences have a way of recurring in the world; yet somehow we find it hard to believe in ones that crash down on our heads from a blue sky. There have been as many plagues as wars in history; yet always plagues and wars take people equally by surprise. (. . .) Stupidity has a knack of getting its way; as we should see if we were not always so much wrapped up in ourselves. (. . .) A pestilence isn't a thing made to man's measure; therefore we tell ourselves that pestilence is a mere bogy of the mind, a bad dream that will pass.

Camus, like his predecessors, could not miss the analogy between plagues and war.

> (p. 39) (. . .) He must fix his mind, first of all, on the observed facts: stupor and extreme prostration, buboes, intense thirst, delirium, dark blotches on the body, internal dilatation, and, in conclusion. . .In conclusion, some words came back to the doctor's mind; aptly enough, the concluding sentence of the description of the symptoms given in his medical handbook: "The pulse becomes fluttering, dicrotic, and intermittent, and death ensues as the result of the slightest movement." Yes, in conclusion, the patient's life hung on a thread, and three people out of four (he remembered the exact figures) were too impatient not to make the very slight movement that snapped the thread.
>
> (p. 40) The carnival of masked doctors at the Black Death; men and women copulating in the cemeteries of Milan; cartloads of dead bodies rumbling through London's ghoul-haunted darkness—nights and days filled always, everywhere, with the eternal cry of human pain.

In every widespread epidemic, the grotesque, the pathetic, inversion, pleasure—first of all charnel, sensual—marries suffering, fear, and terror.

> (p. 63) On the day when the death-roll touched thirty, Dr. Rieux read an official telegram that the Prefect had just handed him, remarking: "So they've got alarmed at last." The telegram ran: Proclaim a state of plague stop close the town.

(p. 77) (. . .) commerce, too, had died of plague.

(p. 80) (. . .) One of the cafés had the brilliant idea of putting up a slogan: "The best protection against infection is a bottle of good wine," which confirmed an already prevalent opinion that alcohol is a safeguard against infectious disease.

Superstitions and magical cures are a big success during an epidemic.

(p. 81) (. . .) One of [the stories] was about a man with all the symptoms and running a high fever who dashed out into the street, flung himself on the first woman he met, and embraced her, yelling that he'd "got it."

Is this not the writer's stage effect? Or the expression of a reality: the morbid desire to share one's pain, forcefully if necessary, with the first stranger to be found, and perhaps infect him? Undoubtedly both. It is well known that in the beginnings of the AIDS epidemic, the stricken voluntarily contaminated others.

(p. 91) (. . .) Rieux had learned that he need no longer steel himself against pity. One grows out of pity when it's useless.

(p. 92) (. . .) These manifestations of public piety were to be concluded on Sunday by a High Mass celebrated under the auspices of St. Roch, the plague-stricken saint.

(p. 93) (. . .) Even Tarrou, after recording in his notebook that in such cases the Chinese fall to playing tambourines before the Genius of Plague, observed that there was no means of telling whether, in practice, tambourines proved more efficacious than prophylactic measures.

Belief, faith, although they did not cure, they did not worsen the sickness—it is like Pascal's wager.

(pp. 122–123) As usual! That was to say the new consignment of serum sent from Paris seemed less effective than the first (. . .). Most of the buboes refused to burst—it was as if they underwent a seasonal hardening—and the victims suffered horribly. During the last twenty-four hours there had been two cases of a new form of the epidemic; the plague was becoming pneumonic. On this very day in the course of a meeting, the much-harassed doctors had pressed the Prefect—the unfortunate man seemed quite at his wits' end—to issue new regulations to prevent contagion being carried from mouth to mouth as happens in pneumonic plague. The Prefect had done as they wished, but as usual they were groping, more or less, in the dark.

In speaking of the authorities, Camus says:

(p. 124)(. . .) What they're short on is imagination. Officialdom can never cope with something really catastrophic.

Most people had little experience of scourges, and collective memory forgets quickly. How could one imagine and live through the unspeakable? Today we have television; but the natural or human dramas and tragedies "only happen to others," don't they?

(p. 131) (. . .) The narrator does not share that view. The evil that is in the world always comes of ignorance, and good intentions may do as much harm as malevolence, if they lack understanding. On the whole, men are more good than bad; that, however, isn't the real point.

(p. 168) (. . .) the ambulances clanging past, sounding the plague's dismal, passionless toxin under their windows.

(p. 179) (. . .) by reason of their very duration great misfortunes are monotonous. In the memories of those who lived through them, the grim days of plague do not stand out like vivid flames, ravenous and inextinguishable, beaconing a troubled sky, but rather like the slow, deliberate progress of some monstrous thing crushing out all upon its path.

(p. 181) (. . .) Indeed, to some, Dr. Rieux among them, this precisely was the most disheartening thing: that the habit of despair is worse than despair itself.

(p. 182) (. . .) For there is no denying that the plague had gradually killed off in all of us the faculty not of love only but even of friendship.

In daily routines of the disease's ravages, the best sentiments of humanity are lost, and this is almost worse than the loss of one's loved ones.

(p. 214) The doctor's hands were gripping the rail of the bed, his eyes fixed on the small tortured body. Suddenly it stiffened, and seemed to give a little at the waist, as slowly the arms and legs spread out X-wise. From the body, naked under an army blanket, rose a smell of damp wool and stale sweat. The boy had gritted his teeth again. Then very gradually he relaxed bringing his arms and legs back toward the center of the bed, still without speaking or opening his eyes, and his breathing seemed to quicken. Rieux looked at Tarrou, who hastily lowered his eyes.

They had already seen children die—for many months now death had shown no favoritism—but they had never yet watched a child's agony minute by minute, as they had now been doing since daybreak. Needless to say, the pain inflicted on these innocent victims had always seemed to them to be what in fact it was: an abominable thing. But hitherto they had felt its abomination in, so to speak, an abstract way; they had never had to witness over so long a period the death-throes of an innocent child.

Infectious diseases do not spare innocent children—on the contrary! we could say. Camus could only be remembering that the recently ended Second World War had killed thousands of children, many more than all the other wars: the great plague, natural, was becoming truly human.

(p. 227–228) At this point Father Paneloux evoked the august figure of Bishop Belzunce during the Marseille plague. He reminded his hearers how, toward the close of the epidemic, the Bishop, having done all that it behooved him, shut himself up in his palace, behind high walls, after laying in a stock of food and drink. With a sudden revulsion of feeling, such as often comes in times of extreme tribulation, the inhabitants of Marseille, who had idolized him hitherto, now turned against him, piled up corpses round his house in order to infect it, and even flung bodies over the walls to make sure of his death. Thus in a moment of weakness the Bishop had proposed to isolate himself from the outside world—and, to and behold, corpses rained down on his head! This had a lesson to us all; we must convince ourselves that there is no island of escape in time of plague. No,

there was no middle course. We must accept the dilemma and choose either to hate God or to love God. And who would dare to choose to hate Him?

The author, not much of a believer if not a nonbeliever, like his character can only be resigned to come back to the Christian roots of his culture: "There was no middle course."

(p. 307) (. . .) but tonight they told not of revolt, but of deliverance. (. . .)

From the dark harbor soared the first rocket of the firework display organized by the municipality, and the town acclaimed it with a long-drawn sigh of delight. Cottard, Tarrou, the men and the woman Rieux had loved and lost—all alike, dead or guilty, were forgotten. Yes, the old fellow had been right; these people were "just the same as ever." But this was at once their strength and their innocence, and it was on this level, beyond all grief, that Rieux could feel himself at one with them. And it was in the midst of shouts rolling against the terrace wall in massive waves that waxed in volume and duration, while cataracts of colored fire fell thicker through the darkness, that Dr. Rieux resolved to compile this chronicle, so that he should not be one of those who hold their peace but should bear witness in favor of those plague-stricken people; so that some memorial of the injustice and outrage done them might endure and to state quite simply what we learn in time of pestilence: that there are more things to admire in men than to despise.

Nonetheless, he knew that the tale he had to tell could not be one of a final victory. It could be only the record of what had had to be done, and what assuredly would have to be done again in the never ending fight against terror and its relentless onslaughts, despite their personal afflictions, by all who, while unable to be saints but refusing to bow down to pestilences, strive their utmost to be healers.

And, indeed, as he listened to the cries of joy rising from the town, Rieux remembered that such joy is always imperiled. He knew what those jubilant crowds did not know but could have learned from books: that the plague bacillus never dies or disappears for good; that it can lie dormant for years and years in furniture and linen-chests; that it bides its time in bedrooms, cellars, trunks, and bookshelves; and that perhaps the day would come when, for the bane and the enlightening of men, it would rouse up its rats again and send them forth to die in a happy city

Camus's novel, on the terrible theme of the plague, is capable of both moving and appeasing the reader. It is indeed the work of a great artist.

Albert Camus, 1913–1960, was also a great journalist and essayist, a great playwright and novelist, and Nobel Prize awardee in Literature. Camus did not die of the plague, nor of tuberculosis, cured with antibiotics, but died at 46 years of age in an automobile that hit a tree—he was not driving.

40.2.4 Cholera, the Blue Plague

Cholera is caused by the *Vibrio cholerae* bacterium, disseminated through the fecal–oral route as a consequence of sewage and fecal contamination of drinking water and food. It is now widely recognized that the disease was endemic in South Asia for at least 2000 years, especially in the delta lands of Ganges in West Bengal and Bangladesh, from which it spread periodically in epidemic form.

Jean Giono, Le Hussard sur le Toit [38]

This French writer (1895–1970) would have been awarded the highest honors and the greatest prizes if he had not voluntarily kept himself isolated from the society of his time. His unwavering pacifism sent him to prison. After the Second World War, he changed the style of his writing from the earthy lyricism of his peasant novels, then his militant works, to a more lively and enthusiastic writing in the remainder of his major novelistic works, what can be called the Angelo cycle from the name of the central character, notably two epic novels, *The Horseman on the Roof* (1951) and *The Straw Man* (1957). Angelo was an aristocratic character, both in background and temperament, but he remains at the edges of society and law, "cultivating the art of not getting fooled" and feels "at the height of happiness" in exercising his freedom [33].

The 1832 cholera epidemic in Provence and elsewhere, one of the first in Europe, is the mold for *The Horseman*. Without it, the novel could not have been constructed as it was, nor even exist. Giono needed to place his heroes and all the characters swarming around them in an extraordinary dramatic context: the terrifying epidemic provided it, and later the war for the continuation of Angelo's adventures. *The Horseman* story can be summarized as follows:

Angelo Pardi, a young Italian, is the only, but illegitimate, son of a great aristocrat woman won over by democratic ideals. She bought him a colonel's commission and he commanded a regiment of horsemen of the kingdom of Piedmont Sardinia. A supporter of the Risorgimento and Carbonarism, in a duel Angelo kills a double agent of the Austrian Empire. Wanted, he hides in France, living in the regions of Aix-en-Provence and Marseille among the exiled Italians. With his affair being sorted out by his influential mother, he decides to go home, but clandestinely. He heads toward the Alps through northern Provence. The reader finds him at the beginning of the novel on horseback, in the middle of the summer, a few leagues from Manosque, where in a small hamlet he discovers all the inhabitants dead of a violent disease, that he later learns is cholera, a new disease that has just landed on the western shores of the Mediterranean. The rest of the novel narrates the hero's adventures and mishaps through the cholera epidemic. He takes refuge in Manosque, living for a time on the rooftops to keep himself away from all human contact. In a house that he believed is deserted, he fleetingly meets a young woman who becomes the heroine of the story: Pauline, wife of the old count de Théus, who is going to join in their castle of the Hautes-Alpes. Angelo withdraws as a gentleman. Later he joins an old nun, who, now that her fellow-nuns have fled, spends her time roaming the streets to wash the dead. He escapes a lynching by the crowd, runs away from the police, and so on. He leaves Manosque, meets a group of his countrymen, one of whom is his foster brother, and then continues his journey alone. He

meets Pauline again at a military roadblock; she accepts his company and protection, but they get arrested at another roadblock. Locked up in a fortress with other people wasting away as they wait in quarantine for the infection, they flee. Later, in a small mountain village where they are soaked by an autumn rain, an old solitary doctor withdrawn to the village, erudite by somewhat misanthropic, takes them in for the night. He tells them of the cholera in a colorful language, full of images. The next morning they leave. At dawn, Pauline collapses, invaded by the disease. Angelo cares for her with every possible means he has garnered. Until this time he had not succeeded in saving a single cholera victim, despite his struggles and the risks he had undergone. Finally he is successful. A few days later they are in Théus. The husband is absent; there is only the elder sister of the count and a few servants. We suspect that Angelo and Pauline can finally love each other carnally, but duty calls her as a wife, and he has married in Italy. They leave each other. Angelo returns to his country. "He was beside himself with joy."

We have extracted from the novel the following quotations, nearly all of them concerning cholera. In the first one, it is a second-class naval doctor at the naval base in Toulon, who has just autopsied a man and diagnosed cholera. His admiral was napping and he must wait before announcing the event. He is thinking about what he will say:

> (pp. 27–28) "What makes me sick," he said to himself, "is being obliged to argue; having to explain matters when, in my fellow from the *Melpomène*, everything is explained clearly, positively, and beyond argument. . . . Bring them the whole thing on a platter, all cut out and prepared ahead of time for the mathematical demonstration of the connections, highly disrespectful toward rank and society, between the distant exaltation of great rivers and the snuffing-out of, day, a hundred thousand human lives. Easier to explain with proofs at one's fingertips.
>
> "Look: the viscous appearance of the pleura—see? And the contracted left ventricle; and the right ventricle full of a blackish coagulum; and the cyanosis of the esophagus, and the detached epithelium, and the intestines swollen with matter that I might liken, sir, in order to facilitate your comprehension of science, to rice-water or whey. Let's open him up, Admiral whose siesta must not be disturbed, let's open up the six feet of the *Melpomène's* quartermaster; dead at noon, Admiral, while you were sipping your coffee and your couch was being prepared; dead at noon, blown up by the Indus Delta and the air pump of the Upper Ganges Valley. Intestines the color of pink hydrangeas; glands isolated, protruding as large as grains of millet or even hempseed; the *plaques de Ryer* gritty; tumefaction of the follicles, what we call psoriasis; vascular repletion of the spleen; greenish soup in the ileocaecale valvule; and the liver marbled: all this in the six feet of the *Melpomène's* quartermaster, stuffed like a stinkball. I'm just a junior officer, Admiral, but I can assure you that we have her a bomb capable of blowing up this country in five seconds like a bloody grenade."

Jean Giono had read all of the documentation on cholera gathered in the nineteenth century. He shows in this scene with the doctor how the art of the writer can transform a precise scientific description into a literary purple passage. There are others throughout the book—*The Horseman on the Roof* is an epic and picaresque novel but also a manual on cholera.

The two bodies in the following passage are those of a young health officer—the one who told him about which disease he was facing—dead in his arms, and a young boy that they had taken in.

> (p. 55) Angelo spent a terrible night beside the two corpses. He was not afraid of contagion. He didn't think about it. But he dared not look at the two faces, as the firelight flickered over them, their drawn-back lips baring jaws with dog's teeth ready to bite. He did not know that people dead of cholera are shaken with spasms and even waver their arms at the moment when their nerves relax, and when he saw the young man move his hair stood on end; but he rushed to massage his legs and continued to massage them for a long time.

Then Angelo is in Manosque. For the first time he sees the death cart pass. The image, the scene is almost a ritual in all the epidemic narratives.

> p. 104 . . . he heard, coming from a side street, the rumbling and creaking of a heavily laden cart. He hid behind a tree trunk and saw two men appear, each holding up a torch. They were escorting a wagon drawn by two strong horses. Four or five other individuals, in white blouses and carrying picks, spades, and more torches, walked beside the wheels. It was a load of coffins, and even corpses simply wrapped in sheets. Arms, legs, heads wagging on long, think, flabby necks, stuck out through the side racks.

Below: At this time—Pasteur is a child—the microorganisms later called bacteria have not yet been recognized as responsible for infectious diseases. But perhaps some were already evoking them. And the novelist has the right to be anachronistic and to place an echo of the debates of the time in his hero's head: literature has often been made in the futuristic and science fiction.

> p. 142. I don't believe the plague's a bearded man, but I'm quite sure it's a little animal, much smaller than a fly and perfectly capable of inhabiting a chair cover or a web of a tapestry.
>
> p. 144. You know it's simply a question of little creatures smaller than flies, which give people cholera.

Later, this image of abandoned children who die in solitude, hiding themselves like animals, is exaggerated, but it was undoubtedly often the case. That too is the horror of the great catastrophes that tormented humanity, a victim of its excesses and its abuses.

> p. 161 . . . a new order (called for the moment disorder) was abruptly organizing life within new horizons. Very few were still able to believe in the virtues of the old cardinal points. They no longer kissed the children. Not to protect them; to protect themselves. Moreover the children all had rigid bearing, monolithic, with wide eyes, and when they died, it was without a word or a groan and always far from their homes, burrowing into a dog kennel, or into a rabbit warren, or hutches, or curled up in the big baskets used for brooding turkeys.

In the worst situations, there are always people capable of practicing compassion and showing respect

> p. 170. They washed abandoned corpses. They could not was all that they found in the night; they lay in every corner. Some were sitting up: they had been deliberately arranged to look like persons resting. The others, thrown down anyhow, would be hidden under filth . . .
>
> They washed only the foulest. They carried them one by one to the side of a fountain. They undressed them. They scrubbed them with plenty of water. They laid them out neatly to be picked up when day came.
>
> p. 174. The nun's chief care was to prepare the bodies for the Resurrection. She wanted them clean and decent for that occasion . . . "I'm a housekeeper; I'm doing my job."
>
> She was very taken aback one night when, after throwing some buckets of water over a corpse, it opened its eyes, then sat up and asked why it was being treated in this way.
>
> It was a man still in the prime of life. He had fainted in a fit of cholera and been taken for dead.

And the animals also change:

> p. 210. It's been noticed, too, that when a horse refuses, it's always a very bad sign for the person or house it refuses . . .The dogs: naturally there's the dogs belonging to all those who have died, and they wander all over the place, feeding on corpses. But they don't die; on the contrary, they grow fat and give themselves airs; they no longer want to be dogs.

In a single colorful sentence, the author expresses the spread of the epidemic that is devouring everything in its passage:

> p. 246. The cholera was now stalking like a lion over towns and woods.

And then derision masks disobedience:

> p. 288. "The stage of absolute funk (they don't beat about the bush). The stage of masquerades, carnivals on the *corso*. People dress up as Pierrots, Harlequins, Columbines, clowns, to get away from death. They wear masks, they put on cardboard noses, false mustaches, false beards, they paint themselves ludicrous faces, they play at '*après moi le déluge*" vicariously. We're right back in the Middle Ages, sir. At every crossroads they're burning straw effigies entitled 'Father Cholera'; they insult it, they laugh at it. They dance around it and then go home to die of fear or diarrhea."

We arrive at the last scene of the novel, with two episodes that deserve to be cited in greater length. First Angelo and Pauline, spending the evening at an old doctor's, a man come out of the worst, who, in his way, tells them of the cholera and what it does to men, their minds, and their bodies:

> p. 394. "It's comical," said the big man in the riding-coat. "We're having an epidemic of fear. Right now, if I were to call a yellow armband `cholera' and make a thousand people wear it, the thousand would die in a fortnight.". . . .
>
> "I've practiced medicine for over forty years. I know perfectly well that the cholera isn't the outcome of pure imagination. But if it spreads so easily, if it has what we call this `epidemic violence,' that's because by the continual presence of death it enhances everybody's huge congenital egoism. People die, literally, or egoism."

The egoism of the patient who ends up reveling in his suffering? Or the egoism that believes it is still healthy and flees the other? But cholera, like all plagues, is not a psychosomatic disease.

> p. 404. While a camel lies in its death throes in the dust of Karakorum, a shopgirl is drinking champagne in a café, a family of crocodiles is descending the Amazon, a herd of elephants is crossing the equator, a llama with a load of borate of soda is spitting in its driver's face on a path in the Andes, a whale is floating between Cape North and the Lofoten Islands, and it's the Feast of the Virgin in Bolivia. The terraqueous globe revolves, heavens knows why or how, in solitude and shadow.

And such is the fate of each one. What good is it believing. . . .

> They had paid him the honor, he believed, of asking him about cholera; he was now ready to reply.
>
> "Enter, let us enter into these five or six cubic feet of flesh about to become cholera-stricken."
>
> "What happened in the beginning? Nobody can tell us."
>
> p. 406. "That is the moment when the cholera victim's face reflects that stupor said to be characteristic. His debilitated joys are terrified today by something other than their own weakness; by some unknown thing from which they flee far beyond true north and are lost to view."
>
> "Fear gives wings and wit. The day darkens. The stupor is not enough: one has to stagger, fall on the spot: at table, in the street, in love, in hate, and attend to far more intimate, personal, and passionate things."

Disease takes you, takes you over, subjugates you. Nothing else counts any more.

> p. 408. Yet people continued to be surprised at the indifference shown by cholera victims to those around them and to the courage and devotion often spent upon them. "In most illnesses, the sick person takes an interest in those who are looking after him. Patients on the point of death have been seen shedding tears over their loved ones or asking for news of Aunt Eulalie. The cholera victim is not a patient: *he's an impatient*. He has just understood too many essential things. He's in a hurry to know more. That's all that interests him, and if you both caught the cholera you'd cease to mean anything to each other. *You'd have found something better.*"
>
> p. 409. "Your cholera victim is prodigiously interested. His one aim from now on is to know more.
>
> "What is it he's feeling? Banal things: his feet are cold. His hands icy. He's cold in what are called the extremities. His blood is receding, rushing to the site of the spectacle."
>
> "Generally, there's nothing to be done. Poultices for wooden legs—as you can imagine, there's an infinite variety of them. Calomel is one. No, I haven't got any. What should I do with it? Sirup of gum perfumed with orange blossom is another. We have a choice between leeches at the anus and bloodletting—one doesn't need much erudition to think of these in such cases. We

can pass from clysters to cachou, from ratany to quinine, mint, camomile, lime, balm. In Poland they give a grain of belladonna; in London two grains of subnitrate of bismuth. Some try cupping the epigastrium, or mustard plasters on the abdomen. Some administer (it's a pretty word) hydrochlorate of soda or acetate of lead."

". . . we keep on looking for a specific capable of neutralizing the toxic attack, according to the formula of learned persons . . ."

All folk remedies or scientists remedies are no more than placebos when confronted with a new evil. Everything is tried but it is too late: The disease has already gone elsewhere.

p. 410–411. "The cholera victim no longer has any face: he has a *facies*—a facies that *could only mean cholera*. The eye, sunk deep in its socket and seemingly atrophied, is surrounded by a livid circle and half covered by the upper eyelid. It expresses either great agitation of the soul or a sort of annihilation. The sclerosis, now visible, is smitten with ecchymosis; the pupil is dilated and will never contract again. These eyes will never have tears again. The lashes, the lids are impregnated with a dry, grayish matter. Eyes that remain wide open in a rain of ashes, gazing at halos, giant fireflies, flashes of lightning.

"The cheeks have lost their flesh, the mouth is half open, the lips glued to the teeth. The breath passing through the narrow dental arcades becomes loud. It's like a child imitating an enormous kettle. The tongue is swollen, flabby, rather red, covered with a yellowish coating.

"The chill, first felt in the feet, knees, and hands, tends to invade the whole body. Nose, cheeks, ears are frozen. The breath is cold, the pulse slow, extremely weak, toward the decline of physiological existence.

"Now in this condition the victim answers with lucidity if questioned. His voice is hoarse but he doesn't wander. He sees clearly, and *from both sides*. When he chooses, it is with full awareness."

p. 411. "Some of my colleagues, who aren't all blind, have spoken of 'choleric asphyxia.' I even thought for a moment that they were capable of understanding and expressing a little more than science whispers into their ears, when they added this charming remark—and how true!—'The air still reaches the blood, but the blood doesn't reach the air.'"

"I've often thought that there is perhaps a moment when the cholera victim suffers, suffers horribly, not in his pride as hitherto (that's what is pushing him on) but, at last, in his love, and this might hold him back on our side."

pp. 412–413. The invalid is in an extreme state of agitation. he tries to rid himself of every covering, complains of unbearable heat, feels thirsty; forgetting all modesty, he flings himself out of bed or furiously uncovers his sexual parts. And yet his skin has turned cold and soaked with an icy sweat, which soon becomes sticky and gives to anyone touching it the disagreeable impression of contact with a cold-blooded animal.

"The pulse becomes more and more faint but it is still very rapid. The extremities take on a bluish tinge. The nose, ears, fingers suffer cyanosis; similar patches appear on the body.

"The emaciation we have noted in the face has extended all over. The skin has lost its elasticity, and *retains the crease if one pinches it*.

"The voice is extinguished. The patient now speaks only in sighs. The breath has a sickening smell, impossible to describe but unforgettable once one has smelled it.

"Calm comes at last. Death is not far off.

"I've seen some come out of this coma, sit up and for several seconds look for air; put their hands to their throats and, with a pantomime as painful as it is expressive, indicate to me an appalling strangling sensation.

"The eyes are turned up, their brilliance has vanished, the cornea itself has thickened. The gaping mouth reveals a thick tongue covered with ulcers. The chest no longer rises. A few sighs. It is over. He knows what to think of the outward marks of respect.

The doctor has given his lesson. Each one can go and rest. Angelo and Pauline take up the road again the next morning, as indicated by their host.

pp. 414–415. It was warm—one of those autumn days that seem like spring . . . A light wind, flecked with cold, gave the air an unparalleled vigor and virtue. Even the mule was happy . . .

The young woman walked along gaily and, like Angelo, kept exclaiming over the clarity of the sky, the beauty of the camellia-colored mountains lost in morning mist, toward which they were heading.

The day passes, a happy one. And, as twilight approaches

pp. 416–417. But she was looking at him stupidly. Before he could cry: "What's the matter, Pauline?" she gave a sort of reflection of a still charming little smile and dropped down, slowly, folding her knees, bending her neck, her arms dangling.

As he rushed to her side, she opened her eyes and plainly tried to speak, but belched out a small flood of white, clotted matter, like rice paste.

This is precisely how the cruel disease takes you down, after having ruminated in you for a long moment without your feeling it.

p. 417. Angelo tore the pack off the mule, spread his big cloak on the grass, and wrapped the young woman in it. He tried to make her drink some rum. Her neck was already as hard as wood and yet it shuddered, as if from tremendous blows struck deep inside her.

Angelo listened to those strange appeals to which the whole body of the young woman was responding.

Here, our hero no longer wants to lose against evil. He rebels. Everything that he has learned through his contact with the epidemic and the dying, and the lesson bestowed by the old doctor, came back to him. He attacks the victim to save her. Because he is a man, and warrior, because she is a woman, and perhaps obedient, it is like rape.

p. 417. He pulled off the young woman's boots. Her legs were already stiff. Her calves were trembling. Her straining muscles protruded from the flesh. From her mouth, still plastered with its flow of rice, came little, shrill moans. He noticed that her lips were curling back over her teeth and that the young woman had a sort of cruel, almost carnivorous laugh on her face. Her cheeks

were fallen in and palpitating. He began to massage her icy feet with all his strength.

. . . He had also to light a fire and heat some big stones.

pp. 418–419. Angelo had placed some large pebbles in the fire. When they were very hot, he wrapped them in some linen and laid them close to the young woman's stomach. But the feet had turned purple. He began to massage again. He could feel the cold fleeing from his fingers and climbing up the leg. He raised the skirts. An icy hand seized his.

"I'd rather die," said Pauline.

Angelo gave some answer, he knew not what. That voice, though a stranger's, put him in a sort of tender rage. He shook off the hand brutally and tore out the laces binding the skirt at the waist. He undressed the young woman the way one skins a rabbit, dragging off the petticoats and drawers bordered with lace. He immediately massaged the thighs but, feeling them warm and soft, he withdrew his hands as though from live charcoal and returned to the legs and knees, which were already in a grip of ice and turning blue. The feet were snow-white. He uncovered the belly and studied it attentively. He felt it with both hands, all over. It was supple and warm but traversed with shudders and cramps. He could see it was inhabited by bluish shapes, swimming about and rising to strike the surface of the skin.

The young woman's groans now came rather loudly with each spasm. They made a continuous complaint, betraying no very great pain but accompanying the deep workings of some sort of ambiguous state, which was waiting, even hoping (or so its seems), for a paroxysm when the cry would become savage and, as it were, delirious. These spasms that shook the whole body recurred every minute, making Pauline's stomach and thighs crack and arch each time, leaving her exhausted under Angelo's hands after each assault.

He never stopped massaging . . . He at once attacked the thighs, which were beginning to be peacock-patterned with blue spots. He renewed the little nest of burning-hot stones around the stomach.

. . . Pauline had begun to foul herself below. He cleaned everything carefully and placed under her buttocks a draw-sheet made of embroidered underclothes that he had taken out of the little case.

"She must be forced to drink some rum," he thought. . .He struggled to unlock the teeth. He succeeded. The mouth opened. "The smell isn't nauseating," he thought, "no, it doesn't smell bad." He poured in the rum, little by little. At first it was not swallowed, but then the alcohol vanished like water in sand.

He automatically raised the bottle to his own lips and drank . . . but said to himself: "And so what?"

The cyanosis seemed to have settled in above the thighs. Angelo energetically massaged the folds of the groin . . . . Her stomach was still shuddering with memories. The moaning had ceased.

p. 420. She continued to disgorge clotted, whitish matter. Angelo noticed a frightful stench spreading. He wondered where it came from.

. . . He could only massage without stopping. His hands ached. He massaged with *eau de vie*. He kept renewing the hot stones. He carefully dragged the young woman as close as possible to the fire.

. . . The absence of hope, rather than despair, and above all physical exhaustion now made him more and more frequently turn to gaze into the night. He was not seeking help but some response.

. . . Yet he never ceased to labor with his hands to bring warmth to that groin at the edges of which the cold and marble hue still lurked.

At length a whole series of little, highly colored, brightly lit thoughts came to him, some of them absurd and laughable, and, at the end of his tether, he rested his cheek on that stomach, now shuddering only feebly, and fell asleep.

. . . It was day.

He could not think what the soft, warm thing was on which his head was resting. He could see he was covered to the chin by the folds of his cloak. He breathed deeply. A cool hand touched his cheek.

"I covered you up," said a voice. "you were cold."

He was on his feet in an instant. The voice was not entirely unfamiliar. Pauline was looking at him with almost human eyes.

. . .At length he had the sense to feel the patient's pulse. It was beating quite strongly and its speed was on the whole reassuring.

pp. 421–422. Angelo remembered the maize water Teresa used to make him take when he was small: apparently it cured everything, particularly dysentery.

. . . The young woman drank greedily, several times. Towards noon the cramps were plainly over.

. . . the young woman announced that she now felt as warm and soft as a chick in its egg.

p. 423. "I'm anxious about you," she said. "I've had cholera, there's no doubt about that.. . . And you, haven't you been rash?"

. . . "Yes, but in these cases the infection shows at once. I've got a night's start on death," said Angelo, "and it won't catch me up."

p. 425. "It is," thought Angelo, "a case of cholera, of course, but a cured one."

. . . They reached Théus two days later, in the evening.

p. 426. This horse gave him matchless pleasure for three days. Her kept thinking of it. He saw himself galloping.

Every evening Pauline put on a long dress. The illness had made her little face sharper than ever. It was as smooth and pointed as a lance-head and, under the powder and rouge, faintly tinged with blue.

"How do you think I look?" she said.

"Very beautiful."

The morning that he left, Angelo right away gave free rein to his horse, which he had himself, every day, fed with oats. It had a swiftness he could be proud of. He saw galloping toward him those rosy mountains, near enough now for him to make out the rising larches and firs on their lower slopes.

"Beyond is Italy," he thought.

He was beside himself with joy.

And so ends *The Horseman on the Roof*. A few years later a third novel of the Angelo trilogy appeared: *The Straw Man*, where Angelo, always as magnificent, continues his epic, his odyssey, no longer in cholera but this time in war and revolution: six of one half a dozen of the other/ same difference!

40.2.4.1 The blue plague and the yellow fever

Barbara Hambly, Fever Season, 1998 [52]

Barbara Hambly is a contemporary American novelist who was trained as a historian. After publishing a few heroic Gothic novels that received critical acclaim, in 1997 she began the saga of Benjamin January—a free black man of nineteenth

century New Orleans. *Fever Season* is the second title of the series, a thriller that plunges the reader into the murky world of the city of lagoons and bayous, both poverty- stricken and brilliant. The action takes place in 1833–1834. For the last 25 years, Louisiana, and particularly the city of New Orleans, has experienced an epidemic of yellow fever, especially during the hot season. That year cholera also broke out.

Also, the sudden emergence of yellow fever on the continent was related to the collapse of the French military personnel sent by First Consul Bonaparte to retake Saint Domingo, where the rebel slaves had taken power. Of 25,000 men, 15,000–20,000 men of the French army perished from yellow fever, with 20 generals out of 23, including the Chief Commandant Leclerc, Bonaparte's sister Pauline's husband. There is no doubt that the epidemic disaster influenced the French decision to sell Louisiana to the United States.

Benjamin January is a dark quadroon, in his forties, free. When he was an adolescent, his mother, a relatively well-off mulatto, maintained, sent him to Paris to study medicine. He would have continued living in France, if his wife, a French mulatto, had not died of cholera the year before, 1832. January thus returns to his home country and family. Forbidden to practice medicine because he was a Black, he gets by as a musician, with a passion for shady and criminal affairs. With the double epidemic ravaging the area, the medical authorities accept January's help at the Charity Hospital, but only at night. Several stories intertwine: a slave fleeing or kidnapped, the mysterious death of a planter, buccaneers and pirates in the bayous and the river, abductions of slaves and free Blacks sold in Missouri, and a large bourgeois house where atrocious crimes are committed. Barbara Hambly brings to life an entire illicit society, both European and African, taken up in the epidemic torment that whips up passions. In this environment and atmosphere, where the famous mulatto Marie Laveau rules, the mistress of voodoo, Benjamin January, having grieved his dead wife, rediscovers the temptations of love in the person of a mulatto young lady, Rose, who runs a small boarding school for young colored girls from good families, refusing that they become like their maintained mothers "placées," as was said at the time) or prostitutes. Barbara Hambly took inspiration from true events reported at the time in the New Orleans and Louisiana gazettes. Her novel is also the sociological study of a changing, disordered world, underscored by the epidemics—and then there are the hurricanes (we are writing these lines as Katrina is raging at the mouth of the Mississippi and its great city, at the end of September 2005; many things have changed little since the 1830s).

Let us cite the following passages from the novel:

> (p. 1, the beginning of the story) In fever season, traffic in the streets was thin. Those who could afford to do so had left New Orleans with the ending of Lent; those who could not had all through the long summer hurried about their business as if Bronze John, as they called the sickness, were a creditor one could avoid if one kept off the streets.

To be better protected against the mosquitos.

> (p. 22) His last patient that day had been a nine-year-old girl who'd walked the twelve streets to the hospital from the levee where she'd been selling oranges. Her mama, she said in English, before delirium claimed her, would whale her for not staying on to finish the day. The child had died before she could tell anyone who her mama was or where that lady could be found.

As always, the image of innocent childhood struck down—although at the time, as in *The Horseman*, many children died, victims of infectious diseases; people were used to it, even if the mother's and father's suffering was real each time.

> (p. 2) The fever had first come to New Orleans in Janauary's sixteenth year.

1809. But it seems that yellow fever had touched Louisiana a few years earlier.

> (p. 4) Like the Americans uptown, the householders here burned piles of hair and hooves from the slaughteryards or smudges made up with gunpowder, to clear the disease-ridden miasma from the air.
>
> p. 5 When Bronze John came calling, a lot of people, no matter how strait their circumstances, came up with the money to remove for the summer to one of the hotels or cottages on the shores of the lake, where the air was cleaner, in Milneburgh or Mandeville or Spanish Fort. Those who hadn't done so from fear of the fever, which came nearly every year—or from the horrible combination of summer decay and summer insects—reconsidered the matter when the first cases of the cholera were diagnosed.

In the nineteenth century as in the Middle Ages, the reaction was always the same: flee the bad air—this was only valid for the well-off or the rich, without really protecting them more than the poor and destitute.

> (p.19) Though Bronze John's hand touched everyone, white, black, and colored, it was mostly the whites who died of it and, of them, more often the whites who'd flocked into New Orleans from the United States—the *rest* of the United States, January corrected himself—or from Europe.

A fair remark showing the different degrees of sensitivity to the infection or of acquired immunity.

> (p. 24) Fever rode the night air, invisible and deadly—that was all that anybody knew of it.
>
> (p. 27) Dome wasting sickness there, thought January, studying her rigid profile with a good physician's eye. Not consumption.

A quiet reminder of that other, slowly evolving infectious disease that was also rife at the time, notably in the Western world.

> (p. 30) A fortress against Bronze John, he thought. Against the cholera. Locked and shuttered, like every other house on the street, in the hopes of thwarting nightbone, drifting enemies no one could see.

Fear of the "bad air" in the ignorance of microbes (but for yellow fever, as for malaria, the link was made with mosquitos: There were mosquito nets around beds and even doors and windows).

(p. 61) Salts of mercury mixed with turpentine have been shown to be of sovereign benefit—sovereign, sir!—in cases of fever, Sanchez retorted. But the dosage must be heroic.! Nothing is of any benefit unless the patient's gums bleed . . .

It is always the same medicine, ridiculed by Molière!

(p. 115) (. . .) Yellow Jack hits quick. (. . .) . . .

Yet another nickname that personified the disease. In a note at the bottom of this page, the author indicates that yellow fever was the other name given to cholera. This is clearly wrong: Cholera was not known in North America until that year of 1833.

(p. 156) In the silence it was easy to believe the disease roved the streets like the angel of death. Easy to half-expect the skeletal white shape of Baron cemetery, the voodoo lord of the dead, coming around a corner in his top hat and his spectacles. (. . .)

Janvier, like all of the colored people of the city, knew his secret myths and rites well. He is in relation with Marie Laveau, the true and famous mistress of voodoo, who was also a healer.

(p. 159) *The disease isn't contagious*, he told himself, slipping from tomb to tomb. He dodged behind one, then another, working his way through the dense-packed mazes. *I've worked among the dying for three months now and I haven't contracted it yet.*

Or the mystery of immunity . . .

(p. 165) January had hunted enough mosquitoes within the tents of mosquito-bar—trying to singe them to death with a bedroom candle where they clung to the gauze without immolating the house—to know that nobody in Louisiana would leave the bar untied.

(p.173) January thought suddenly of all those houses standing locked and empty, nd of the fear that fueled drinking, and the drinking that fueled violence in an already violent town.

(p. 230) . . . but plague or no plague, January felt he must breathe clean air, or die. Smudges of lemongrass and gunpowder burned on the gallery over the water, keeping at bay the mosquitoes which, though fewer than in town, could be found even along the lakeshore.

(p. 306) "Would you believe it ladies?" he turned to take in the dozen or so who clustered at his heels, "when I came down to the city last summer, at the very landing where I boarded they loaded a matched team of white horses whose cost alone could have provided a dozen beds for those wretched sufferers dying in the alleys of this city during the pestilence? It isn't God who sent the fever to punish mankind, ladies; it is Man who brought it upon himself, with sheer, greedy neglect of his fellow man."

An evangelist preacher who no longer believes in divine will! He knows the human kind too well.

(p. 345) "One thing I've learned," January said with a smile, "love is beyond comprehension. Anyone can love anyone. It's like the cholera.

(p. 348) They spoke of the epidemic, and of why the fever might come in the summertimes and not the winters, and why not every summer; of why sometimes cures seemed to work—even onions under the bed—and why sometimes they did not; of the white ghost-crabs that scurried in the retreating scrum of the surf, and of pirate treasure and hurricanes. "I've watched the winds and the clouds here," said Rose, "and the winds and air in the marsh. It feels different there, but I can't say why it's different, what is different about it. There has to be some way of identifying what it is. Everyone talks about the miasma of sickness, but it's only a guess, you know. There has to be a way of making it visible, like a chemical stain turning the color of water."

"This will blow past, like a hurricane. It always does."

"And like a hurricane," said Rose softly, "it will leave wreckage, and that long tedious season.

The character, like her author, knows what she is talking about, in the country of hurricanes and fevers, of poverty and the music in dance and festivities.

40.2.4.2 Love in the Time of Cholera [80]

Gabriel Garcia Marquez, A Colombian writer in Spanish who was born in 1928, Gabriel Garcia Marquez is qualified in a dictionary as a "fantastic storyteller." Undoubtedly. But we could add that he is an extraordinary novelist whose name will remain in the history of literature; perhaps the world's greatest writer of the end of the last century. The Nobel Prize he was awarded in 1982, several years before the publication that we will now discuss, was greatly deserved, not always the case. It should be remembered that he is also the author, among other talented works, of *One Hundred Years of Solitude* and *Chronicle of a Death Foretold*. En ce qui concerne "L'Amour au Temps du Choléra," le quatrième de couverture de l'édition que nous avons en main dit: "L'auteur (. . .) donne libre cours dans ce roman à son génie de conteur, à la richesse de son imagination et à l'enchantement baroque de son écriture. " - ô combien !

Love in the Time of Cholera is the story of three characters: a woman and two men. It takes place over more than 80 years, from the middle of the nineteenth century, for the most part in a city, a Colombian port giving onto the Caribbean, probably Cartagena, as well as in a few other places and on the Magdalena River.

Two teenagers love each other with an absolute love that remains epistolary and platonic while waiting for the time to marry. The young man, Florentino Ariza, is poor but destined for a great inheritance; the girl, Fermina Daza, comes from the wealthy bourgeoisie. When he returns from Paris, where he has perfected the art of medicine, the young doctor Juvenal Urbino, the most brilliant match of the city and the province, instantly falls in love with Fermina, and he is the one she chooses. Florentino lives with his failure for more than half a century, devoting himself to work and women through carnal love, but he remains true to Fermina: He stays

a bachelor, never doubting that one day she will be his, and he will be hers. Urbino's life proceeds as was right and proper; he is a great doctor and professor and a high notable of the city; she a great lady with her home life and social life, attentive to her charities and her children; she is also a woman with an artistic spirit, as liberal as her husband. Doctor Urbino ends up dying, stupidly, septuagenarian, still vigorous, and healthy. Florentino Ariza resumes his long-interrupted correspondence with Fermina Daza, the widow Urbino. She accepts the game, and then accepts to receive her old sweetheart. They celebrate their friendly and still chaste reunion by taking a cruise on the river on board the largest and most luxurious ship in the country, a ship Florentino has owned for years. After a last hesitation, it is on board ship that their bodies finally come together, regaining the sexual fire of their youth in their decrepitude, a curious couple of lustful old people.

In the setting and in the background of the story of this flamboyant trio of very romantic characters, the entire life of an old city and an ex-colonial country unwind before our eyes in the transformations of social and technical modernity, as in North America and Europe: steam power, natural gas, electricity, the automobile, the airplane, and radio all appear. Political life was meant to be democratic, but changes in orientation and personalities between liberals and conservatives are sorted out by popular violence and sometimes with cannon fire and by armed force; this often happens somewhere not far from the coast, where commerce predominates. Finally, there is cholera, whose great epidemics are in decline but which remains endemic, like a threat that is continually renewed. The novel is a sort of baroque opera: joyous, lively, where the outdoors are celebrated in song and is, despite death so often present, a great hymn to life, on which the yellow flag of cholera (once the flag of the plague) seems to wave, the one that will lead to the unexpected ending of the novel, burlesque as much as grave.

The choice of our quotations undoubtedly does not give sufficient homage to Garcia Marquez's unparalleled talent and style. But at least half of the novel would have to be quoted. Let us suffice with the passages that exemplify our subject best.

> (p. 3, the start of the novel) It was inevitable: the scent of bitter almonds always reminded him of the fate of unrequited love. Dr. Juvenal Urbino noticed it as soon as he entered the still darkened house (. . .). Jeremiah de Saint-Amour, disabled war veteran, photographer of children, and his most sympathetic opponent in chess, had escaped the torments of memory with the aromatic fumes of gold cyanide.
>
> (. . .) At one window the splendor of dawn was just beginning to illuminate the stifling, crowded room that served as both bedroom and laboratory, but there was enough light for him to recognize at once the authority of death.
>
> (p. 17) At nightfall, at the oppressive moment of transition, a storm of carnivorous mosquitoes rose out of the swamps, and a tender breath of human shit, warm and sad, stirred the certainty of death in the depths of one's soul.

Dawn, twilight, and death. These two passages, a mere 20 pages apart, give an idea of the author's suggestive power.

> (p. 43) Soon after he had completed his course of specialized studies in France, Dr. Juvenal Urbino became known in his country for the drastic new methods he used to ward off the last cholera epidemic suffered by the province. While he was still in Europe, the previous one had caused the death of a quarter of the urban population in less than three months; among the victims was his father, who was also a highly esteemed physician.
>
> (p. 63) All that was needed was shrewd questioning, first of the patient and then of his mother, to conclude once again that the symptoms of love were the same as those of cholera.

This curious and striking parallel is indeed Garcia Marquez! Barbara Hambly (see above), writing later, "borrowed" it from him, not a great sin; we know that artists in all the arts copied one another, even plagiarized, cheerfully, or at least took inspiration from their predecessors. It is possible that there are social or picturesque observations of epidemics that have been repeated since the time of Thucydides.

> (p. 108) He tried to impose the latest ideas at Misericordia Hospital, but this was not as easy as it had seemed in his youthful enthusiasm, for the antiquated house of health was stubborn in its attachment to atavistic superstitions, such as standing beds in pots of water to prevent disease from climbing up the legs, or requiring evening wear and chamois gloves in the operating room because it was taken for granted that elegance was an essential condition for asepsis. They could not tolerate the young newcomer's tasting a patient's urine to determine the presence of sugar, quoting Charcot and Trousseau as if they were his roommates, issuing severe warnings in class against the mortal risks of vaccines while maintaining a suspicious faith in the recent invention of suppositories. (. . .)
>
> (pp. 108–109) His obsession was the dangerous lack of sanitation the city. He appealed to the highest authorities to fill in the Spanish sewers that were an immense breeding ground for rats, and to build in their place a closed sewage system whose contents would not empty into the cove at the market, as had always been the case, but into some distant drainage area instead. The well-equipped colonial houses had latrines with septic tanks, but two thirds of the population lived in shanties at the edge of the swamps and relieved themselves in the in the open air. The excrement dried in the sun, turned to dust, and was inhaled by everyone along with the joys of Christmas in the cool, gentle breezes of December. Dr. Juvenal Urbino attempted to force the City Council to impose an obligatory training course so that the poor could learn how to build their own latrines. He in vain tried to stop them from tossing garbage into the mangrove thickets that over the centuries had become swamps of putrefaction, and to have them collect it instead at least twice a week and incinerate it in some uninhabited area.
>
> pp. 109–110 He was aware of the mortal threat of the drinking water. The mere idea of building an aqueduct seemed fantastic, since those who might have supported it had underground cisterns at their disposal, where water rained down over the years was collected under a thick layer of scum. Among the most valued household articles of the time were carved wooden water collectors whose stone filters dripped day and night into large

earthen water jars. To prevent anyone from drinking from the aluminum cup used to dip out the water, its edges were as jagged as the crown of a mock king. The water was crystalline and cool in the dark clay, and it tasted of the forest. But Dr. Juvenal Urbino was not taken in by these appearances of purity, for he knew that despite all precautions, the bottom of each earthen jar was a sanctuary for waterworms. He had spent the slow hours of his childhood watching them with an almost mystical astonishment, convinced along with so many other people at the time that waterworms were animes, supernatural creatures who, from the sediment in still water, courted young maidens and could inflict furious vengeance because of love. (. . .) And so it was a long while before he learned that waterworms were in reality the larvae of mosquitoes, but once he learned it he never forgot it, because from that moment on he realized that they and many other evil animes could pass through our simple stone filters intact.

For a long time the water in the cisterns had been honored as the cause of the scrotal hernia that so many men in in the city endured not only without embarrassment but with a certain patriotic insolence. When Juvenal Urbino was in elementary school, he could not avoid a spasm of horror at the sight of men with ruptures sitting in their doorways on hot afternoons, fanning their enormous testicle as if it were a child sleeping between their legs. It was said that the hernia whistled like a lugubrious bird on stormy nights and twisted in unbearable pain when buzzard feather was burned nearby, but no one complained about those discomforts because a large, well-carried rupture was, more than anything else, a display of masculine honor. When Dr. Juvenal Urbino returned from Europe he was well aware of the scientific fallacy in these beliefs, but they were so rooted in local superstition that many people opposed the mineral enrichment of the water in the cisterns for fear of destroying its ability to cause an honorable rupture.

Or the art of transforming the image of malevolence into a buffoon's painting.

Impure water was not all that alarmed Dr. Juvenal Urbino. He was just as concerned with the lack of hygiene at the public market, a vast extension of cleared land along Las Animas Bay where the sailing ships from the the Antilles would dock. (. . .) Set on its own garbage heap, at the mercy of capricious tides, it was the spot where the bay belched filth from the sewers back onto land. The offal from the adjoining slaughterhouse was also thrown away there – severed heads, rotting viscera, animal refuse that floated, in sunshine and starshine, in a swamp of blood. The buzzards fought for it with the rats and the dogs in a perpetual scramble among the deer and succulent capons from Sotavento hanging from the eaves of the market stalls, and the spring vegetables from Arjona displayed on straw mats spread over the ground.(. . .)

The entire passage is a humorous class on the need for hygiene and asepsis and a demonstration that the environment created by humans is the main cause of infectious diseases: the anthropic factor.

(p. 111) There graves were dug deep enough to bury the dead on three levels, without delay and without coffins, but this had to be stopped because the brimming ground turned into a sponge that oozed sickening, infected blood at every step.(. . .)

(p. 111–112) From the time the cholera proclamation was issued, the local garrison shot a cannon from the fortress every quarter hour, day and night, in accordance with the local superstition that gunpowder purified the atmosphere. The cholera was much more devastating to the black population, which was larger and poorer,(. . .)

(. . .) Dr. Marco Aurelio Urbino, the father of Juvenal, was a civic hero during that dreadful time, as well as its most distinguished victim. (. . .) Years later, reviewing the chronicle of those days, Dr. Juvenal Urbino confirmed that his father's methodology had been more charitable than scientific and, in many ways, contrary to reason, so that in large measure it had fostered the voraciousness of the plague. He confirmed this with the compassion of sons whom life has turned, little by little, into the fathers of their fathers, (...) and it was with justice that his name was found among those of so many other heroes of less honorable wars.

(. . .) When he recognized in himself the irreversible symptoms that he had seen and pitied in others, he did not even attempt a useless struggle but withdrew from the world so as not to infect anyone else. Locked in a utility room at Misericordia Hospital, deaf to calls of his colleagues and the pleas of his family, removed from the horror of the plague victims dying on the floor in the packed corridors, he wrote a letter of feverish love to his wife and children, a letter of gratitude for his existence in which he revealed how much and with fervor he had loved life. (. . .)

(. . .) (p. 113) Until then Dr. Juvenal Urbino and his family had conceived of death as a misfortune that befell others, other people's fathers and mothers, other people's brothers and sisters and husbands and wives, but not theirs.

(p. 114) Cholera became an obsession for him. He did not know much more about it than he had learned in a routine manner in some marginal course, when he had found it difficult to believe that only thirty years before, it had been responsible for more than one hundred forty thousand deaths in France, including Paris. But after the death of his father he learned all there was to know about the different forms of cholera, almost as a penance to appease his memory, and he studied with the most outstanding epidemiologist of his time and the creator of the cordons sanitaires, Professor Adrien Proust, father of the great novelist. So that when he returned to his country and smelled the stench of the market while he was still out at sea and saw the rats in the sewers and the children rolling naked in the puddles on the streets, he not only understood how the tragedy had occurred but was certain that it would be repeated at any moment.

The moment was not long in coming. In less than a year his students at Misericordia Hospital asked for his help in treating a charity patient with a strange blue coloration all over his body. Dr. Juvenal Urbino had only to see him from the doorway to recognize the enemy. (. . .)

(p. 115) From that time on, and well into this century, cholera was endemic not only in the city but along most of the Caribbean coast and the valley of the of the Magdalena, but it never again flared into an epidemic. The crisis meant that Dr. Juvenal Urbino's warnings heard with greater seriousness by public officials. They established an obligatory Chair of Cholera and Yellow Fever in the in the Medical School, and realized the urgency of closing up the sewers and building a market far from the garbage dump.

In these pages, Garcia Marquez brilliantly describes the changing paradigm in medicine in terms of infectious diseases, in the second half of the nineteenth century. This son who takes over for his father, but in transforming the tool and the vision of the goal, is like a parable of the passage of ancient to modern medicine by looking for the intimate secrets of these patients and by imposing hygiene and asepsis.

> (. . .) (p. 218) Tránsito Ariza used to say: "The only disease my son ever had was cholera." She had confused cholera with love, of course, long before her memory failed. But in any event she was mistaken, because her son had suffered from six blennorrhagias, although the doctor had said they were not six but the same one that reappeared after each lost battle. He had also had a swollen lymph gland, four warts, and six cases of impetigo in the groin, but it would not have occurred to him or any man to think of these as diseases; they were only the spoils of war.

Another passage that is not only a purple patch but also it shows that venereal diseases, in men's minds, in the minds of the Western men of the time, were experienced like a malevolence that signified a certain panache, the most esteemed, that is to say sexual, making an institution of the brothel and its prostitutes.

> (p. 232) Someone asked what terrible illness would dare to attack a woman with so much power, and the answer he received was saturated with black bile:
>
> (p. 299) "A lady so distinguished could suffer only from consumption."

Tuberculosis was not only a disease of the rich, far from it. But this disease of slow progression was easier to live with, as it were, than any other. As the author writes below, it was a "fashionable" disease.

After several episodes returning to the protagonists' lives, this is the finale, the two lovers' cruise on the river, like a honeymoon that they never want to end. And to accomplish this, they find a way.

> (pp. 342–343) The only thing that would allow them to bypass all that was a case of cholera on board. The ship would be quarantined, it would hoist the yellow flag and sail in a state of emergency. Captain Samaritano had needed to do just that on several occasions because of the many cases of cholera along the river, (. . .)After all, everyone knew that the time of cholera had not ended despite all the joyful statistics from health officials. (. . .) If such things were done for so many immoral, even contemptible reasons Florentino Ariza could not see why it would not be legitimate to do them for love. (. . .)
>
> The New Fidelity weighed anchor at dawn the next day, without cargo or passengers, and with the yellow cholera flag waving jubilantly from the mainmast. (. . .)
>
> (. . .) (p. 345) For they had lived together long enough to know that love was always love, anytime and anyplace, but it was more solid the closer it came to death. (. . .)
>
> "It is going to be like dying," she said (speaking of their return)
>
> (. . .) in conclusion, after a string of barbaric curses, was that he could find no way out of the mess he had gotten into with the cholera flag. (the ship had been put in quarantine)
>
> Florentino Ariza listened to him without blinking. Florentino Ariza l'écouta sans ciller.and he said: "Let us keep going, going, going, back to La Dorada." (. . .) The Captain looked at Fermina Daza and the first glimmer of wintry frost. Then Ariza, his invincible power, his intrepid whelmed by the belated suspicion that it was life that has no limits
>
> "And how long do you think we can keep coming and going?" he asked.
>
> Florentino Ariza had kept his answer ready for fifty-three years, seven months, and eleven nights. (the time since they were teenagers that he had declared his love to Fermina)
>
> Forever," he said.

40.2.5 The White Plague

The swift, impetuous vigor, the lightning speed and ferociously lethal nature of epidemic infectious diseases such as the plague, cholera, yellow fever, and certain strains of typhus should not conceal the slower but equally fatal power of other diseases such as leprosy, tuberculosis, or syphilis. The latter two diseases had particular psychological effects on artists, influencing the work of their numerous victims (HIV AIDS is too recent to estimate its "artistic" impact; however, in literary texts on the subject, influences appear from texts inspired by the two older diseases that are reasonably comparable for their physiological and psychological effects).

Tuberculosis was the modern artist's disease *par excellence*. It was linked to various artistic movements, in particular Romanticism. If art was influenced by a single infectious disease, tuberculosis was in this sense exemplary.

> In the 19th century its pulmonary form, consumption, became one of the most harmful diseases in developed societies. It is a metaphor which characterizes the dominant pathology of the time. [43]

Tuberculosis was the motif and/or the theme of a fertile literary output, fanning the flame of its creativity, which is presented in the first part of the present chapter.

Let us return to some of it:

> In the 19th century) In Mediterranean countries, (tuberculosis) was believed to be contagious (. . .) In France, this hypothesis was vigorously rejected, and that of hereditary transmission was preferred. No detail was spared in the description of the characteristics of children and adolescents who suffered from the disease: slender, delicate beings with fine features and beautiful hair, who did little physical activity, but who had a lively intelligence and were precociously mature, with a talent for art and a particular ability to understand and to feel.

Yes, artists! Moreover:

> (. . .) In this Romantic period, consumption struck heavily among writers, poets, musicians, painters, actors. [98]

40.2.5.1 Paul voivenel

Paul Voivenel, a doctor who was the author of literary essays and criticism, wrote:

> All these famous tuberculosis sufferers, Mozart, Millevoye, Sciller, Maurice de Guérin, Schubert, Chopin, Laforgue, Novalis, Glatigny, Mérimée, Rachel, Marie Bashkirtseff, Bastien Lepage, Chekhov, Hugues Rebell, Albert Samain, suffered to a certain extent from what Camille Mauclair called "The Disease of the Infinite" for which Watteau, himself consumptive, painted the definitive setting in *The Embarkation for Cythera*.

Shelley can also be added to the list (although he died in a shipwreck), as well as Keats, and many others.

> The metaphysical ingenuity of Novalis, the feverish tenderness of Chopin, the sometimes tragic smile of Laforgue, the idealistic beauty of Mozart, the pastoral passion of Schubert, all that is found in the country that Watteau drew out of nature, deep within which is heard, with indescribable emotion, the murmur of *Invitation to the Voyage*.
>
> Tuberculosis hollows out and ennobles the face, and refines the soul: "Hence, in Watteau, the son of a roofer, even before he could see it himself, an idealization of luxurious society formed. Neither prolonged observation nor birth could have bestowed upon him such a unique ease in the expression of what was exquisite, refined. The painter drew from nature, but the artist imposed his preconceived vision and this vision was simply the desire for a paradise of melancholy and love." [101]

It is curious, and even remarkable, that this apparent relationship between tuberculosis and art was still so strongly emphasized in the early twentieth century, whereas it had become known, even in France, that tuberculosis was a contagious disease, transmitted from one individual to another, and not hereditary: The artists mentioned above did not become such, or develop such a talent, because of their illness. However, because of the limits it forced on them, the disease could have highlighted their ability for sensitive, emotive, and artistic expression. Tuberculosis did become a subject for artistic creation, in particular in literature.

> A painless disease, tuberculosis leaves the intellectual, emotional, and creative qualities intact. The long period of physical inactivity encourages introspection, imagination, and projects in an atmosphere of sympathetic attention from the patient's family circle, faced with the tragic destiny of young men and women who had been promised a brilliant future, or who were afflicted at the height of their glory. As their health deteriorates, death is waited upon with fatalism, passively (. . .) A poetic, romantic death, like that of Marie Duplessis, immortalized as La Dame aux Camélias (Camille), like that of Chopin, Marie Barshkirtseff and so many others. [99]

40.2.5.2 La dame aux camélias, or the fate of a consumptive Camille (La Dame aux Camélias) is one of the most famous characters in Romantic literature—a dying Romanticism, as the work represents the swan song of the movement. In the story of this woman, tuberculosis appears to play only a supporting role; however, it is an allegorical role, the disease being the cause of the physical and material decline of the heroine, and then of her redemption through Love (with a capital L), before she is cut down in the prime of her brilliant and captivating youth. The author, Alexandre Dumas fils, called his work a novel, but apart from a few details such as names and the final episode, it was not a work of fiction. Dumas fils recounted faithfully his passionate love affair (at least from his point of view!) with one of the most admired and sought-after courtesans of 1840s Paris: Marie Duplessis, whose fame he passed down to posterity under the name Marguerite Gauthier, la Dame aux Camélias.

Marie Duplessis was born as Rose Alphonsine Plessis on January 16, 1824, in a Norman village. She was 15 years old when her father took her to Paris and placed her with a milliner of the Palais-Royal—a milliner who was also a go-between. In addition to her beauty, the young girl had intelligence and character. At 16, she took her freedom; at 17 she began to be well known and sought after in the courtisan milieu. Then she had her coming out in the "beau monde" by becoming the mistress, very well maintained, of a very rich old man (every day he sent her armfuls of camelias, this sumptuous but unscented flower). From then on she could choose her lovers, wealthy. She was 20 years old when Alexandre Dumas fils, the same age, fell in love with her, who responded to his love, by discouraging him by her practices and her lifestyle, before convincing him that their liaison cold go nowhere. He broke it off. Marie Duplessis died of consumption a year later, at barely 23 years of age, on February 3, 1847. When Dumas fils met her, she was already experiencing coughing fits, spitting up blood (3 years was the average reprieve accorded by the disease at this time).

A few months after the young woman's death, as if to atone for having abandoned her, Dumas fils told the story of their love as a novel, in which "he expressed his shame and the beauty of his mistress's soul, portraying her as a sinner redeemed by love who sacrificed both her fortune and her vanity so as not to harm the man she loved" [71]. It was an immediate success, and even more so a few years later when the author turned the novel into a tragedy for the stage. Transposed from reality to fiction, the heroine no longer simply dies from the distressing disease but instead succumbs almost magically to the malady of Love. The final scene, when Marguerite lies dying in the arms of her lover and declares her passion for him, has drawn floods of tears from generations of spectators. Yes, without tuberculosis there would never have been a Marguerite Gauthier, and her model, Marie Duplessis, would only have been one among many in the troop of courtesans, those diamond-eating tarts—who grew up as simple working-class girls—before whom princes, ministers, and financiers of bourgeois, conquering Europe, bowed down and in whose arms they swooned, until August 1914.

Almost all art forms have since taken up the character of La Dame aux Camélias, from academic painting to music, the latter through the opera composed by Verdi based on a libretto by Francesco Maria Piave, just after the appearance of the play, under the title of La Traviata, in which Marguerite is called Violetta. Up to the present day, the greatest opera singers have sought to play the role, just as on the stage, and

on screen a host of actresses have played Marguerite Gauthier. An identical theme, with a heroine dying of consumption, can be found in numerous literary and/or stage productions, many of which take place in an artistic setting. The best known of these is Henri Murger's "Scènes de la Vie de Bohème" that was turned into an opera, Puccini's "La Bohème," by film directors.

Hence, tuberculosis had found its key character, its heroine. During this time, through the "silent" disease, each year slow death cut down millions of children and adults from the lower working classes of Europe (and elsewhere) in overcrowded city slums. Few artists recorded this physiological misery, which was only brought into "social" literature at the end of the nineteenth century, in the work of Emile Zola, for example. It was also at this time that sanatoriums, establishments for rest and specialized care in the treatment of tuberculosis, appeared. The living conditions and the life of their residents became literary subject matter between the two World Wars.

40.2.5.3 Literature of the sanatorium From the beginning of the twentieth century, the first hand accounts gathered in sanatoria revealed that this sort of voluntary prisoner who lived there made up a particular and eccentric cultural group, followers of what today could be called a subculture, even a counterculture, in which forced inactivity developed the intellect and the imagination, and in which temptations and desires formed an important component of their lives, the potential for acting out these desires being great. In 1902, a novel, Les Embrasés by Michel Corday, dealt with the emotional life of tuberculosis patients living in the sanatorium. It was the starting point for an investigation led by a group of tuberculosis specialists. "The nearly unanimous conclusion was that `the rousing' of the tuberculosis sufferers was not so much related to a direct or indirect action of the Koch bacillus on the nerve centers regulating the reproductive activity of consumptive patients as to the inactivity that is imposed upon them." [100]

The major literary work inspired by life in the sanatorium is Thomas Mann's The Magic Mountain (1924) [80]. The story takes place a little before the First World War [64]. A young German, Hans Castorp, the reflection of the author's personality, from a family of the ruling class but deprived of his parents as a child, goes into a Swiss sanatorium shortly before becoming an engineer. He joins the life of the sanitorium and shows himself to be receptive to the disease. His mind undergoes a sort of mutation, opening up to previously unknown areas of interest, while falling in love with a Russian resident. He immerses himself in the study of human biology, extending to the phenomena of spiritual life. He is assisted in his studies by two other residents, Settembrini, an Italian writer and an intellectual idealist, and Naphta, both a Jesuit and a communist, from a Jewish family, a young, ambitious and self-centered dialectician with pre-Facist ideas, who confronts Settembrini in vehement debates attended by Hans Castorp who attempts to summarize the two men's opposing views. The morbid nature of the disease plays a role in Hans Castorp's reflection, but he feels that reason should be an instrument for life. "Man, in the name of goodness and love, should not allow death to gain a hold over his thoughts." "An elementary truth in authentic humanism," comments L. Liebritch. Castorp leaves the sanatorium and his "magic mountain" more or less cured, only to be called up for combat where death catches up with him. The Magic Mountain is a great novel of disease experienced—to be cured of it—in the very particular setting of sanatoria, the very exterior and interior design of which could be seen and read as a work of art.

Let us note a few passages from the novel [55]:

> (p. 486) Because our interest in death and illness is nothing but a way of expressing an interest in life – just look at how the humanistic faculty of medicine always addresses life and its illness so courteously in Latin. (. . .)
>
> (p. 98) A human being who lives as an invalid is only body, and that is the most inhuman of debasements – in most cases, he is no better than a cadaver.
>
> (p. 456) this much was certain: illness meant an overemphasis on the physical, sent a person back to his own body, cast him back totally upon it, as it were, detracted from the worthiness and dignity of man to the point of annihilation by reducing man to mere body.
>
> (p. 12) Hans Castorp suddenly stopped in his tracks, mesmerized by a perfectly ghastly noise he heard coming from beyond a dogleg in the hall (. . .) it was a cough (. . .) but sounded as if someone were stirring feebly in a terrible mush of decomposing organic material. (. . .) a cough devoid of any zest for life or love (. . .) as if you were looking right down inside and could see it all – the mucus and the slime

The Magic Mountain is perhaps the most complete work written on disease as an expression of life able to lead to death.

Tuberculosis in general and its treatment in the sanatorium gave birth to a literary genre where introspection was as important as the story. Moreover, one could almost say that there was a sanatorium literature, just as there was a train station literature and add that it is through tuberculosis that the experience of disease was taken up by literature. In a relatively abundant production, this is well illustrated by the French novel written by Paul Gadenne, Siloé (Gallimard, 1941), where the character experiences his illness as a coming-of-age journey or adventure [65]. In a commentary on his book, the author writes

> Disease, in tearing a man from his environment, his routines, represents an incredible chance for renewal. This physical misery that drops on him like an experimental chamber where, in unheard-of temperatures, under pressure as yet unknown, will develop a new man.

The analyst of the novel specifies "This is a quest in which Nature and Woman play the role of mediators, the girl Ariane and the torrent Siloé symbolize purity and authenticity" [67].

He (Simon, the hero of the novel that illness is initiating) said that, "life in the depths of himself was like a river that is foaming, like water that is boiling, like a pod that is bursting." (. . .) And "that the response to our questions could be provided to us as much by a cataclysm as by a doctor." [66]

Disease provides knowledge, because health "is the absence of psychological experience of the relation of thought to the body." [29]

Non-Western—but westernized—poets and novelists, such as the Japanese writers Sakutaro and Kajaii, both tuberculosis patients, felt and expressed nearly identical sentiments and thoughts in their experience of their disease, notably the diseased human being's relation with nature [17]. Shinoda makes this remark (ep. 167): "He (Kajii) stares at the bule because he must keep still, doing nothing, while sunbathing (this was the only therapy against consumption at the time)." Could sunbathing for tuberculosis patients be the beginnings of tanning in light-skinned people? Or of nudity as an offer to the sun like living art?

However, more than the disease itself perhaps, it may well be the therapeutic treatment through the retreat imposed in the sanatorium that developed or reinforced the artistic expression in some of these consumptive patients.

40.2.6 Syphilis

> from her eye, Black sky, spawner of hurricanes, drank in Sweetness that fascinates, pleasure that kills.
>
> Charles Baudelaire, The Flowers of Evil [3]

M.D. Gmerk and J.C. Sournia write about the nineteenth century:

> Since 1800, syphilis has had clinical patterns that it did not have in the past. (. . .)
>
> With alcoholism and tuberculosis, syphilis plays a major role in the collective imagination and in the literature of Western countries; it is seen as one of the causes of the biological decline of modern man. It is true that social habits, notably prostitution, encourage the spread up to the highest levels of society. [44]

Is this a true change in the disease? Or has medicine achieved a clearer vision of the disease? It was indeed studied, spoken of, feared, and this is said, even more than during the two previous centuries that followed the enormous and terrible initial outbreak of the disease in the sixteenth century. In people's minds, the disease was related to sexual practices, let us say diversified and/or "debauched." But until then it had not been highly visible and was silent in artistic, graphic, or literary expression or creation, insofar as the censure was strict.

Through writing or erotic or pornographic drawing, the libertine philosophy went a long time back to Antiquity. However, it grew during the era of XIV and developed even more throughout the eighteenth century, the Enlightenment, to reach, just before the French Revolution, the extremes of the Marquis de Sade, and, more or less sinisterly or cynically, outside the context of crime, the more joyous if not poetic expression of a knight from Nerciat. But, whatever the authors, allusions to sexually transmitted diseases, syphilis first and foremost, were nearly nonexistent or showed up at best as slap and tickle jokes, amusing things to say, or in colorful language. Let us add that public pornographic expression, and even private, was forbidden, censured, fought against, and therefore confidential, which did not prevent its clandestine production from enjoying success and being sold "under one's coat."

That is not to say that erotic and libertine expression was freer in the nineteenth century—on the contrary, it could be said. But many artists, in all the arts, succumbed ardently to the deadly carnal, sensual, and sexual temptations and pleasures, in particular with prostitution; moreover, writers and other artists frequently broached the subject in private as well in their journals or in letters. Finally, of those texts that were not purified or destroyed by prudish and ashamed heirs, many were analyzed, commented, and published in the twentieth century in literary journals or in editions of the author's complete works.

In actual fact, nearly all of these gentlemen—and a few ladies as well—had a superactivated sexual life (skirt chasers, as we say, bohemians in every sense of the word), oriented toward local exoticism—prostitution—or, more in the literal sense, beyond the Mediterranean Sea (later toward the far away Orient, the Pacific, "the islands"), outside of traditional moral religious and cultural precepts, which, we can now say, was their right. However, this sexual life had its dangers because it was infected by venereal diseases, in particular syphilis, which was widely disseminated by urbanization, professional or occasional prostitution that at times was vagabond, in a bourgeois industrial society that was in full expansion, where misery was also sexual. In this favorable environment, syphilis produced tremendous ravages despite preventive and treatment measures on the part of the authorities, developed to curb what was, with tuberculosis, one of the two main pandemics, or rather endemics, of the modern world.

These artists knew that syphilis was a contagious disease through sexual intercourse, a disease that killed, even if slowly (10–20 years survival at the time), which led to a filthy death by decimating the mind as well as the body, a terrifying disease that was experienced as such, a disease that in men accentuated the fear of women (of prostitutes—or all women were prostitutes in one way or another!), reaching hatred in the prevailing misogyny—fear but also a morbid attraction. A disease that, finally, contributed to the reflection on illness in general, on the state of illness, on life and death, as was the case with tuberculosis in another manner.

Therefore, with the accounts written and preserved from those concerned, many commentaries were written concerning such and such an author or such and such a work, aided by the revelation of the intimate turpitude of each one. But this remained confidential, academic, the subject of literary, scholarly research expressed in the pages of more or less

obscure journals, rarely—or noted episodically here and there—in the pages of the daily press or magazines read by the public. France's international reputation—the reputation of Paris as the capital of vice and depravity, elegant or vulgar (London and other large cities were not set straight on the issue)—was what it was and what it remains today; the subject is very French. But it was not until the end of the twentieth century that a widely circulated book was published, well documented and, in addition, adroitly written. It is entitled, simply, Artists in the Bordel [71]. The period covers roughly a century since Romanticism. The author has called a subchapter "The specter of the mors syphilitica," which begins thus [75]

> The veneral evil was the great fear of the nineteenth century. Striking sexual intercourse performed outside of the sacred and asepticized relations of conjugality, much like AIDS today, syphilis attested to the guilt inherent to purely carnal pleasure, this deadly pleasure. The symptomatic consequences of this disease, with its chancres and its putrid decay, worsened the malediction even more [74].
>
> Murger is dying of a disease that rots everything alive, a senile gangrene complicated by charcoal, a horrible thing where you fall to pieces. I was cutting his mustache the other day, and his lip came away with the hairs," wrote the Goncourt brothers, on that 28th of January 1861, with no idea that a few years later the younger of the two would succumb to this atrocious disease. Besides, thinking about it, they added, this death "looks like a death from Writing. It looks like the death of the Bohemian, this decomposing death – where everything is intertwined, from Murger's life and that of the world he painted: a profusion of nighttime work, periods of misery and periods of feast, poxes poorly cared for, the heat and cold of existence without a home, eating but not dining, small glasses of absinth that console the pawnbrokers; everything that wears us down, everything that burns us, everything that kills us, life rebelling against the hygiene of the body and the soul, which means that a forty-two-year old man leaves life in tatters because he hasn't the vitality to suffer and complains of only one thing: the odor of rotten flesh that is in his room. It is his own [45].
>
> Like a dreadful end-of-the-century forewarning prophet, Murger was dying in the odor of syphilis, as is said of dying in the odor of sanctity.

Adds from Manéglier, citing a current author [103].

> Thus sexuality was cursed; and the prostitute who embodied it better than anyone else inevitably became an abyss of perdition. For the artist it was a double danger: the danger of exhaustion by frenzied fornication and the danger of death by invasion of the treponema [74].

It should be remembered that Henri Murger was the charming author of Scenes from the Life of a Bohemian, where, when the characters die, it is from tuberculosis . . .

And yet, the medical school and its most dignified representatives warned against the excess of fornication, which, beyond the risk of the dreadful disease "could only result in limiting the force of creation," wrote the learned ones in all seriousness.

> Pulling himself out of Hélène's arms, the young model for the Schio Massacres, Delacroix noted that she had taken a part of his day's energy. The only thing left for him to do was clean his brushes and go and lounge around the cafes and wait for tomorrow to arrive. [75]

Artists believed him, as Flaubert wrote to his friend Bouilher: "Poor wretch, if you spill your come in such a way. You will have no more for your inkwell. And that is the true vagina of you men of letters." [35] But they kept at it, the devils! And there was sin: one was punished by where one sinned: disease as punishment, an old refrain. A German theologian wrote in 1519:

> Tell those who keep the Holy Scripture that the pox came from the wrath of God and that God would punish our miserable life and torment it. [59]

In the medical descriptions of the nineteenth century, the horrors described joined the horrors of war. But, a certain Guy de Maupassant exclaimed in a letter written in 1877:

> I've got the pox! Finally! The true pox! Not some contemptible clap, not the pure cleric, not the bourgeois dandy, no, the great pox, the one that killed King Francis I. And I am proud, zounds! And I scorn the bourgeois above all. Hallelujah, I've got pox, and so I am no longer afraid of catching it. [76]

Oh the braggart, the boaster! who would die a stunned madman 15 years later.

However, his elder and friend, his mentor, Norman as he was, the great and good Gustave Flaubert, expressed no less sharply in his letters his wounds from carnal love that he received with his companions on his voyage to the Orient:

> (. . .) in Beirut I picked up (I realized it in Rhodes, homeland of the dragon) seven chancres, which ended in melting into first two, and then one. (. . .) I suspect a Maronite of having given me this gift, but perhaps it was a little Turkish girl. Is it the Turk or the Christian, which of the two? problem? what a thought!! here is one of the aspects of the Orient question that the Revue des Deux Mondes did not suspect. (. . .)

And, speaking of Maxime du Camp

> That's the third pox that he has caught since we have been on the road. Nothing is better for the health than traveling.
>
> (. . .) my wretched dick, know that it is cured for the moment. There is hardly more than a slight induration, but that is the scar of the brave. It heightens it with poetry. You can see that it is experienced, that it has had its misfortunes. It gives it a fatal and cursed air that should please the thinker.

Flaubert was no fool, but he preferred mockery—making fun of himself—joking to mask his worry. He came out of it rather well, but died of syphilis at the age of 59, or of an excess of heavy Norman cooking; why not both? Perhaps Gabriel Garcia Marquez took inspiration from a few illustrious predecessors, such as this one, to write the passage that

we have cited from Love in the Time of Cholera. At any rate, it is with such anecdotes that "syphilis was transformed into the disease of geniuses" [77]. Ménéglier adds on the next page:

> This black Romanticism would make syphilis a very singular disorder. At this time at the end of the nineteenth century, it would not take long to find an esthetic quality in it. The esthetics of the horrible and of the well-hung seduction. Behind woman's burning and coveted flesh, the Mors Syphilitica, lurking in the depths of the vagina like a moray at the back of its hole, lay in wait for the foolhardy and fascinated it. (. . .)

And a few nude barbarian beauties, adorned in paints, jewels, or some transparent veil, painted by Gustave Moreau, inspired Jean Lorrain, a writer of the epoch that succumbed to the poisonous charms, to write:

> I love her air of plague victim and black virgin got up in satin, like the ones we see in the chapels in Spain. How lovely she would be as a Madonna of the Terror-Stricken, in one of Goya's corteges of the penitents! It is Our Lady of the Seven Lusts . . . [68]

Let us note in passing that our gentlemen rarely had words of passion for the women, most often anonymous, who contaminated them. They were the vector, seductive, irresistible. And the men, were they not as much so when they fornicated, in exchange for a few cents, with very young girls, sometimes hardly nubile? Well! Our remark is perhaps anachronistic (But what of AIDS today?). Shall we say that in the nineteenth century, venereal diseases—with syphilis as the conqueror—was the artists' lot. One had to truly be a saint to escape it. Artists are not saints. Did their all-out, unbridled copulations encourage their talents and their genius? It is doubtful if one considers Victor Hugo who passed away at an old age, despite his ardent sexuality in the last part of his life. How many artists died after long and horrid agonizing in their middle age, before exhausting their talents? The list is long, suicides of high-risk love. There were few of them who used their disease to transcribe into their works the physical and mental torments that it put them through. Among the French writers, let us choose three; the last one, nearly our contemporary, was not contaminated but lived a part of his life in fear of syphilis, a fear that showed through his writing.

Guy de Maupassant (1850–1893) is quite exemplary. He was already infected by syphilis (the "pox" as he calls it; see the quotation above), when he was recognized as a talented writer by his friends, the critics, and the public, with the publication of the short story, Boule de Suif, the big-hearted prostitute who gave herself to the enemy to save her traveling companions, on the road in a diligence, who scorned her (Maupassant wrote a number of stories staging prostitutes and brothels, reflections from his unremitting practice of dangerous venal love, revealing, however, a certain respect (tenderness?) for these women, a few madams included). It is interesting to note that all his work was written while he was syphilitic and that this civil servant, filled with a joie de vivre in the existence that he shared in another fashion between his friends, sailing, and easy women, and that he knew how to sharply but pertinently denounce the flaws of bourgeois, even aristocratic, society (he was born in a castle), which he considered hypocritical and merciless. He continued to write, despite the psychological problems brought on by his cruel disease. And then it was the apotheosis, or the fall. In 1887, he wrote one of his strongest texts, La Horla, where the luminous and morbid, horrible hallucinations of his character are the transcription of his own torments. Then, 5 years of decline, desperation, rot, and finally internment before death relieved him.

Alphonse Daudet (1840–1897). His Letters from My Windmill and Tartarin of Tarascon were read by every schoolchild in France and perhaps they still are. Le dictionnaire Robert writes of this Provençal:

> Whether it be fanciful tales or novels of social commentary, in Daudet there is always a taste for the truth, tempered by a delicate sensitivity and a constant compassion for the weak.

Undoubtedly. But the schoolchildren did not realize—nor did most teachers—that behind the "sensitive" writer existed a man who was obsessed with copulation throughout his entire life. In Paris, an active and honored member of the artists' guild, he openly joined in the escapades and debaucheries of his companions, all as well known or better known than he was. Other than that, he was a good husband and father, but he did not escape syphilis. At more than 50 years of age, he experienced the tertiary and final stage of the disease, and he had the courage to keep a journal of his illness, what it subjected him to, and the thoughts and feelings that the disease and treatment fed his mind with. He began a journal: The Land of Pain. He died without being able to publish it.

This volume "Alphonse Daudet, la Doulou, 1887–1895 and Le trésor d'Arlatan: 1897" (illustrated, t. 17 Edit. Ne Varietur, Paris, Librairie de France, 1930) is the original edition of The Land of Pain (La Doulou). The study and publication of communications from a conference, "Ecriture et Maladie," a book that we have cited in the section on tuberculosis and contains a paper by Michael Worton entitled "Narration, Dialogue et Diagnostic: le cas Alphonse Daudet" (pp. 107–119), whose subject is La Doulou.

A few quotes [26]

> (p. 13) torture . . . there are no words to express it, only howls of pain could do so. Are words actually any use to describe what pain (or passion, for that matter) really feels like? Words only come when everything is over, when things have calmed down. They refer only to memory, and are either powerless or untruthful.

In his journal, Goncourt writes on May 31, 1886

> He [Daudet] also talks to me about the sort of vacillation that bromide brought to his memory, forcing him, he said, to grab hold of the edges of memories; and on this subject, he sends forth a curious observation, affirming that Flaubert's struggle

with words must have come from the enormous mass of bromide that he had absorbed.

Daudet writes:

(p. 41) Effect of morphine.
Wake up in the night, with nothing beyond a mere sense of existing. But the place, the time, and any sense of self, are completely lost.
Not a single idea. Sense of EXTRAORDINARY moral blindness. [25]
(. . .)
(p. 4–5) 'The ship has fouled' is the nautical phrase. I need some such term to describe the crisis I find myself in. (. . .) I'm going down. [27]

What else can be said?

Julien Green (1900–1998), the writer with dual American-French nationality, who wrote in French, accompanied the last, tormented century throughout its duration. The harshest and most severe diseases—tuberculosis and syphilis—that touched his close relatives haunted Julien Green, who was raised by his mother until, as a young adolescent, he lost her: a puritan who had a phobia of the body and sex, a mother traumatized by losses in her family and who wanted to protect her youngest from being soiled by infectious disease. He was a contemporary of the pseudo-victory over infectious diseases and then of their return, notably the sexually transmitted diseases, augmented by AIDS. Green's entire body of largely autobiographic work revolved around these themes, from the novels to the voluminous journal that he published over more than half of his life. The phobia of the body, the phobia of bodily contact that runs the risk of disease, appears throughout his work.

She regretted that she hadn't given Germaine a kiss, or rather, that she hadn't been able to give her a kiss, because at the moment when she saw her arms reach out to her, a feeling of irrepressible horror made her return to her room. Perhaps, indeed, a single kiss was enough to communicate to her this disease that had afflicted her sister. Admittedly her maiden sister had assured her that she wasn't contagious, but isn't that how all the diseased spoke? [46]

All diseases, in particular syphilis, can only be divine punishment.

I cannot, however, hide the gravity of the risk that I ran, that I am now telling the doctor about, but according to him I am suffering from no more than what he calls syphilophobia and he insists that it is a passing obsession. [47]

Elsewhere we find

(. . .) to allude to disease and death is, as it were, to increase their prestige (. . .). In a sort of anonymity, death seems to me much more imposing than if it were presented with a name borrowed from medical jargon [48].

Finally

The greatest modern scourge, the most recent of the nightmares that terrifies physical love, that terrifies the flesh, AIDS. . .[49].
Diseases stemming from bestiality, syphilis and AIDS, would lead some to believe in a curse, but what do they know about what God thinks? [51]

Thus, Julien Green, homosexual but spared from syphilis and AIDS, completes the circle of disease. Anne-Cécile Pottier-Thoby comments

Disease appears more than ever like an irrevocable component of the human condition. Give in to the world and its temptations lead one there, resisting leads to depression. But whatever face it presents, behind disease emerges the fear of death. [50]

40.2.7 Smallpox, Ebola, and Other Pestilences

40.2.7.1 Smallpox Choderlos de Laclos, *Les Liaisons Dangereuses* [61]; Presentation

(Letter CLXXIII, p. 389) The same person who gave me these details told me that Madame de Merteuuil was attacked the following night by a very violent fever, which, it was thought at first, must be the effect of the terrible predicament in which she had found herself; but since last night it has become known that confluent smallpox *(NB: confluent smallpox is the most harmful, contrary to discrete smallpox, because the closely spaced pock marks cover the entire body)* of a particularly malignant type has declared itself. It would really, I think, be fortunate for her if she died of it.
(Lettre CLXXV, pp. 391–392) Madame de Merteuil's destiny, my dear and worthy friend, to have been fulfilled. It is such that her worst enemies are divided between the indignation she merits and the pity she inspires. I was quite right to say that it would perhaps be fortunate for her if she died of the smallpox. She has recovered, it is true, but horribly disfigured; more than anything by the loss of an eye. As you may imagine, I have not seen her again, but I am told that she looks truly hideous
(p. 392) The Marquis de—, who never loses an opportunity to be spiteful, said yesterday in speaking of her 'that the disease has turned her inside out and that her her soul is now visible on her face'. Unfortunately everyone thought the observation very just. (. . .)

For centuries, smallpox sowed death or, in the survivors, the marks of its pustules, throughout the Old World: Asia, Europe, and Africa. Then the conquerors of the European peninsula unknowingly transported it to the New World. Smallpox—as well as a few other infectious diseases, unknown in the West—wrought devastation as soon as the first bearded White men landed. The decimation of populations in just a few decades, particularly in the Caribbean and Central American, was unprecedented, wiping out between 50% and 95% of the indigenous population, depending on the colonized regions. Indian painters in Mexico represented smallpox victims. As a result, Black Africans were imported for labor—yet another story—except that the slaves perhaps exported yellow fever

to the tropical Americas. Later, smallpox was even used as a biological weapon to reduce the bothersome Indians who refused to submit to the new order. Then the conscience of the conquerors evolved, at least officially in North America. The American president, Thomas Jefferson, a man of the Enlightenment if ever there was one, chose Captain Meriwether Lewis to head the 1803–1806 expedition sent by the Congress of the United States on Jefferson's request to explore the mysterious lands of the west to the Pacific recently bought from Napoleon in the Louisiana Purchase. Lewis was charged with the mission of taking the smallpox vaccine to immunize the Indian tribes that he encountered: For trade, fair or not, there is a need for consumers, not dead people. It seems that Lewis and his men had few occasions to carry this out; perhaps vials were lost, broken, or their contents spoiled. But the fact remains that roughly 30 years later, smallpox sprang up again along the Missouri River and west of it, exterminating entire Indian tribes.

There were a few White witnesses of this ultimate tragedy caused by smallpox. A naturalist scholar, the greatest of the ornithologists, a reputed painter of animals, and author of a remarkable comprehensive survey, *The Birds of North America*, John James Audubon (Jean-Jacques Audubon, born French in Louisiana), traveled in the American far west during these years. In his journal, later published, he reports what witnesses told him of the fatal epidemic [2]. His tale is not a masterpiece of literature, but travel literature was greatly appreciated by the public of Western culture between the eighteenth and the middle of the twentieth centuries. In relation with the theme of this chapter, we believe Audubon deserves to be cited:

> (p. 613) Early in the month of July, 1837, the steamer "Assiniboin" arrived at Fort Clark with many cases of small-pox on board. (. . .) The pestilence, however, had many victims on the steamboat, and seemed destined to find many more among the helpless tribes of the wilderness. An Indian stole the blanket of one of the steamboat's watchmen (. . .), wrapped himself in it, and carried it off (. . .). Mr. Chardon offered a reward immediately for the return of the blanket. (. . .)

But the Indian quickly died.

> "They moved en masse, to confront the awful catastrophe that was about to follow. (. . .) and the small-pox had taken such a hold upon the poor Indians, and in such malignant form, that they died oftentimes within the rising and setting of a day's sun. They died by hundreds daily. (. . .) Men shot their wives and children, and afterwards, driving several balls in their guns, would place the muzzle in their mouths, and, touching the trigger with their feet, blow their brains out.
>
> (. . .) a young man, covered with the eruption, and apparently on the eve of death, managed to get to a deep puddle of mire or mud, threw himself in it, and rolled over and over as a Buffalo is wont to do. The sun was scorching hot, and the poor fellow got out of the mire covered with a coating of clay so as to render it like unburnt bricks, and as he walked or crawled along towards the village, the mud drying and falling from him, taking the skin with it, and leaving the flesh raw and bleeding, he was in agony, and besought those who passed to kill him; but, strange to say, after enduring tortures, the fever left him, he recovered, and is still living, though badly scarred. (. . .)
>
> This is a mere sketch of the terrible scourge which virtually annihilated two powerful tribes of Indians (. . .) The mortality, as taken down by Major Mitchell, was estimated by that gentleman at 150,000 Indians, including those from the tribes of the Riccarees, Mandans, Sioux and Blackfeet (. . .) He concluded by assuring us all that the small-pox had never been known in the civilized world, as it had been among the poor Mandans and other Indians. Only twenty-seven Mandans were left to tell the tale; they have now augmented to ten or twelve lodges in the six years that have nearly elapsed since the pestilence. ≫" [2]

In this story based on eyewitness accounts, one must allow for the Davy Crockett-type inventions, most particularly on the number of victims. It nevertheless remains true that the Mandans and other tribes nearly all disappeared. This occurred 168 years ago. But there are still Plains Indians in North America.

After his return, Audubon little by little shut himself up in silence and died a few years later, about to turn 62 years old, probably of Alzheimer's disease.

When the microbes of fiction save humanity: Herbert Georges Wells (1866–1946) was a pioneering science fiction author using the fundamentals of science (time travel, nuclear conflict, and genetic manipulations), who also attempted to show the positive aspect of scientific and technological knowledge. In *War of the Worlds*, H.G. Wells showed the invasion of the planet Earth by Martians, totally foreign to humanity, who used the Earth and men as the biological fermenting agents required for survival. Powerless, the people are subjected to the invader who destroys them. When a saprophyte bacterium from Earth turns out to be infectious and lethal for the Martians, they are destroyed in a few hours and humanity is saved until the next encounter that Wells plans.

> No one would have believed, in the last years of the nineteenth century, that human affairs were being watched keenly and closely by intelligences greater than man's and yet as mortal as his own; that as men busied themselves about their affairs they were scrutinized and studied, perhaps almost as narrowly as a man with a microscope might scrutinize the transient creatures that swarm and multiply in a drop of water. [105]

40.2.7.2 Reality, fiction, science fiction, or from reality to its frontiers The two World Wars, including the period between them, veiled to a certain degree the impact of infectious diseases, despite the shock of the Spanish flu of 1918–1919 and the sudden attacks of plague, cholera, typus, and yellow fever here and there. Advances in vaccines continued and, between the wars, the antibiotics penicillin and Sulfamide were discovered, which in the 1940s and 1950s brought hope of wiping out all of the infections that had tormented humanity for thousands of years. Vaccinations plus antibiotics equals victory over infectious disease; they did not cure the disease of war, but the gods cannot be expected to

deliver everything at once. The Wisemen of the UN promised peace for 1000 years, if not eternally; in the meantime, a cold war was better than a hot war.

The use of the theme of infectious diseases and/or epidemics did not disappear so easily in literature, as we have seen with Camus's *The Plague*, Giono's *The Horseman on the Roof*, or in the great novels of adventure and love such as *The Rain Comes*, or the introspective novels written by tuberculosis sufferers expressing their sentiments toward their disease. As for the mislaid or assassin microbe, it appeared little or not at all in European detective fiction, a genre that had taken its place since the end of the nineteenth century, or was developing with a more radical and dark mode in the United States. Despite the example of H.G. Wells, the unknown microbes coming from far away, invaders were not yet the key characters in science fiction. And to hell with the venereal diseases that spread from person to person, but that a few pills or tablets or shots in the buttocks would quickly eliminate.

Speaking of the 1960s and 1970s in terms of revolution is perhaps exaggerated, but speaking rather of a period of cultural liberation, first of all sexual, goes without saying. Let us kiss, let us embrace, let us all copulate without hang-ups or risks; long live pleasure, enjoyment, and happiness (the famous search for happiness has finally succeeded); thank you Doctor Freud, Doctor Kinsey, and a few others. Ladies you have been liberated to the greater advantage of your pleasure, first of all the pleasure of the gentlemen.

The return of microbes to literature comes through the vector of science fiction, or rather anticipation à la Jules Verne. In 1969, a young doctor, just out of Harvard Medical School, Michael Crichton (we now know his famous career since then as a novelist and Hollywood scriptwriter) published a novel that could be qualified as a thriller of the near future, entitled *The Andromeda Strain* [23]. This is the year of the first man on the moon. The novel is a great success.

In this novel, an inhabited space ship crashes in Arizona; a microbe escapes that kills a few hundred inhabitants in a village in just a few minutes: Death takes them in their occupations, movements, or gestures of the moment, and only an old man and a baby survive. A cell of four eminent researchers from different disciplines in biology is created at the site and kept protected. They examine the facts, collect samples, and take the two survivors, locking themselves into a secret, multilevel, and underground laboratory of a base in Utah, a laboratory constructed for just such an incident that would introduce an extraterrestrial microbe to Earth: the Wildfire plan. Most of the novel recounts the event and the search to explain and find an answer over 5 days. The tale precisely describes the origins and organization of the Wildfire plan and recounts the adventures of the progression in the four researchers' knowledge from the underground fortress and their debates. The technical and scientific explanations are detailed by the author. Finally, one of the researchers thinks he has found the solution, but he makes a mistake in the follow-up test, and they are confronted with the risk that terrible agent may escape. The alert sets off the countdown of the laboratory's destruction by a nuclear device kept for this purpose. One of the men suddenly understands that the mysterious organism has mutated. Another manages to stop the countdown and humanity has been saved.

Crichton, in a style that is both simple and not excessive, succeeds in maintaining the reader's curiosity and tinting it with dread. The theoretical and technical content is good, interesting, on the whole understandable, and plausible. In addition, the author had obtained first hand knowledge from research centers in microbiology and space techniques from NASA, indicating that the possibility of an extraterrestrial contamination was taken seriously from the beginning of space flight. Crichton was well informed (some would say too well).

> (p. 189.) In fact, man lived in a sea of bacteria. They were everywhere—on his skin, in his ears and mouth, down his lungs, in his stomach. Everything he owned, anything he touched, every breath he breathed, was drenched in bacteria. Bacteria were ubiquitous. Most of the time you weren't aware of it.
> (. . .)
>
> A man easily killed by bacteria was poorly adapted; he didn't live long enough to reproduce. A bacteria that killed its host was also poorly adapted. Because any parasite that kills its host is a failure.
>
> (p. 222) Most of this work was to lead nowhere. They knew that, and accepted it in advance. As Stone was fond of saying, scientific research was much like prospecting: you went out and you hunted, armed with your maps and your instruments, but in the your preparations did not matter, or even your intuition. You needed your luck, and whatever benefits accrued to the diligent, through sheer, grinding hard work.
>
> (p. 225) True, every living thing on earth had at least some proteins-but that didn't mean life elsewhere had to have it.
>
> (p. 226) This was not an academic matter. Biology, as George Wald had said, was a unique science because it could not define its subject matter. Nobody had a definition for life. Nobody knew what it was, really.
>
> (p. 235) Perhaps the human brain had become a kind of dinosaur for man and perhaps, in the end, would prove his downfall.
>
> Already, the brain consumed one quarter of the body's blood supply.
>
> (p. 273) Sir Winston Churchill once said that "true genius resides in the capacity for evaluation of uncertain, hazardous, and conflicting information." (. . .)
>
> One is reminded of Montaigne's acerbic comment: "Men under stress are fools, and fool themselves."

Crichton tells the story of Kalocine, a drug, an experimental chemical product developed by Jensen Pharmaceuticals in spring 1965, that was supposed to cure all cancers.

> (p. 298) February 1966, a pilot clinical trial was undertaken. It involved twenty patients with incurable cancer, and twenty normal volunteers from the Alabama state penitentiary. All forty subjects took the drug daily for one month. Results were as expected: normal subjects experienced unpleasant side effects, but nothing serious. Cancer patients showed striking remission of symptoms consistent with cure.

On March 1, 1966, the forty men were taken off the drug. Within six hours, they were all dead.

(p. 301) It was then that he realized that he, too, was scared. Scared to death. The words came back to him.

Scared to death.

Somehow, that was the answer.

p. 302. He smiled.

"I think we can be fairly confident that the organism will move into the upper atmosphere without causing further difficulty on the surface, so there's no problem there. And as for us down here, we understand what's happening now, in of the mutations. That's the important thing. That we understand.

"Understand," Hall repeated.

"Yes," Stone said. "We have to understand."

The Andromeda Strain was at the origin of a Romantic and cinematographic fashion, with stories that were more fantastic than scientific, deviating toward gruesomeness and terror. However, it was a time when infectious diseases were on the way to being conquered, or were officially vanquished, so why bring them out or use them as a dramatic springboard in entertainment or more serious works?

The Marburg Laboratory accident in 1967 as well as the Ebola epidemics in the Democratic Republic of Congo and Sudan in 1976 brought the discovery of the *filoviruses*, but this was not widely reported by the media, and therefore went unnoticed by the general public. *Filoviruses* were only the last, or rather the next to last members in a procession of hitherto unheard-of microbes causing serious disease and a more or less high percentage of deaths that had been revealed since *Junin* in the 1930s in Argentina to *Machupo* in Bolivia at the end of the 1950s and a few others such as *Hantavirus* in Korea. As had been predicted by Charles Nicolle, the *Fate of Infectious Diseases* was indeed that they would come back in the future. Ebola had barely gone out after its brief outburst that HIV began its ravages in secret. The shock of AIDS seemed to anesthetize the creative minds and consciences. Literature and film took time to seize upon the theme of AIDS. However, Ebola and HIV shook researchers who, as the 1980s progressed, defined the concept of disease, microbes, new viruses, more exactly as *emerging*.

Ebola alerts occurred. In 1992, the national press in the United States revealed the 1989 affair of the pet store in Reston, Virginia, and showed that the administration and virologists had been able to hide since then. One year later, a good novelist, Richard Preston, wrote and published *The Hot Zone* a fictional story of the discovery of Ebola and its possibly dramatic consequences, a great success with the public, with translations in a number of countries. (In 1988, Richard Preston also published *Cobra Event*, the story of a bioterrorist attack on New York by an unknown virus. Then in 2002 came *The Demon in the Freezer*, an investigation into bioterrorist risks.) In 1994, the Hollywood film *Outbreak* came out, which had repercussions in the film industry as significant as *The Hot Zone* had in literature. In the news that year, a French virologist, a specialist in hemorrhagic fevers (an author of this chapter: JPG) working at the Yale Arbovirus Research Unit at Yale University, was accidentally infected with the yet unknown Brazilian virus *Sabia* that he was studying. He was saved in only a few days, but the case increased the media ballyhoo. Finally, that same year, a journalist and science columnist working for *New York Newsdays* and other national media, Laurie Garrett, published *The Coming Plague*, subtitled: *Newly Emerging Diseases in a World out of Balance*, a gripping survey that tells the story of new infectious diseases cropping up over the last several decades. Novels and films—even more so television films—multiplied. In literature, among other more or less minor works, let us give as examples the creations on our subject of three renowned American writers who deserve their success and who are specialized in thrillers and detective puzzles.

Robin Cook. This ex-surgeon began writing thrillers around 1980. His stories take place in the areas that he knows best: healthcare, medicine, and the hospital. He took up our subject in 1995 with the title *Outbreak* (the novel was quickly made into a television film). The story involves a sort of violent black plague that seems to have come from Africa, which was first unveiled in a Los Angeles medical center. The investigator makes a frightening discovery. In 1997, Robin Cook pursues the same theme of an unknown bacterium, but through science fiction, presenting *Invasion*: an extraterrestrial civilization "bombs" the Earth with small, seemingly metallic objects that sting people and take hold of them, inoculating them with a bacterium that awakens and modifies a dormant virus, present since the beginning of life in all living things. The process kills the weakest of them and leaves the survivors in a state of euphoria that subjects them to a mysterious superior power.

In 1999, this author returned to a more plausible (and therefore more cautionary) reality with *Vector*. The story takes place in New York: militants of white supremacy wish to punish those responsible for the cultural and social decadence of the country through an attack that would kill great numbers of innocent people (reminiscent of the Oklahoma City bombing that was set off by a truck bomb). The means: spread a lethal microbe in an office building. They meet a taxi driver, a Russian emigrant—what luck!—a technician who survived the Sverdlovsk catastrophe in the USSR (today Ekaterinburg) where a biological weapons factory exploded, releasing a large amount of anthrax. This gives us the report of the April 2, 1979, Sverdlovsk catastrophe, made public for the first time here, slaughtering the population. The taxi driver joins the project; he sets up a high-protection laboratory in his basement to produce several kilos of anthrax and botulinum toxin. He manages to cultivate a small amount of anthrax. It is tested by mailing the white powder to several strangers. The project develops, but the tenacity of two pathologists stops the catastrophe at the last minute.

p. 126. He went on to describe the visit he and Steve had made to the Jacob Javits Federal Building that morning. He told Yuri that

it was set up perfectly to put the bioweapon in the HVAC duct. "Will you need an aerosolizer?" Yuri asked. "No, not if the weapon comes in a fine powder," Curt said. "We'll use timed detonators to burst the packaging. The circulating fans will do the rest."

p. 127. (. . .) "That's what we're fighting against. We know we'll have some civilian casualties in the struggle. It's to be expected. But it's the government we're targeting."

"There are no civilians in my war," Yuri said. "That's why I want the laydown in Central Park. With a proper wind vector it will take out a large swath of the city. I'm talking about hundreds of thousands of casualties or even millions, not thousands. That's what a weapon is supposed to do. Hell, for your narrow objective you could use a regular old bomb."

For those who read *Vector* after September 11, 2001, the story written 2 years before by Robin Cook leaves one thinking, reality joining fiction: letters containing anthrax sent by mail . . .

Patricia Cornwell. Who has never heard of, or read, this American novelist, a writer since the 1990s of detective novels that are globally successful thrillers? Novels whose main character, the heroine, is a woman, a chief pathologist for the state of Virginia, in Richmond (after nearly a dozen books, the author has to quit her job to continue working as a freelance investigator). In 1997, Cornwell published *Unnatural Exposure* [19]. The heroine must face a serial killer, but the point of the story for us is that the author presents a return of a form of smallpox, nearly 20 years after it has been officially eradicated, a form similar to monkeypox. The heroine is contaminated and isolated in a specialized center. As for all her books, Cornwell is highly informed on the subject, on both the science and technical fronts.

p. 195. [20] after all the murder and madness I had seen in my career, it would be a disease that quietly killed me in the end. I never knew what I was exposing myself to when I opened a body and handled its blood and breathed the air. I was careful about cuts and needle sticks, but there was more to worry about than hepatitis and HIV. New viruses were discovered all the time, and I often wondered if they would one day rule, at last winning a war with us that began with time.

p. 210 (. . .) BL-4 meant scientists doing open war with Ebola, Hantavirus and unknown diseases for which there was no cure.

p. 253. "Dr. Martin," I said. "Do monkeys get monkeypox or are they just the carrier?"

"They get it and they give it where there is animal contact, as in the rain forests of Africa. There are nine known virulent poxviruses on this planet, and transmission to humans happens only in two. The variola virus, or smallpox, which, thank God, we don't see anymore, and molluscum contagiosum."

p. 337 (. . .) They cuffed Dr. Phyllis Crowder after she collapsed to the floor. An ambulance transported her to Sentara Norfolk General Hospital, where twenty-one days later she died, shackled in bed, covered with fulminating pustules. She was forty-four.

Patricia Cornwell is well read. She knows that bad women, the sinners, are punished by smallpox, whether they die or not, by ugliness or rottenness, like Madame de Merteuil in *Les Liaisons Dangereuses*, or Zola's *Nana*.

Tony Hillerman. Almost as well known as his famous countrywoman, this writer of classic detective novels began his literary career late. A native of Oklahoma, the American with European roots was a journalist in his home state, then in Texas, before working and settling in Santa Fe and Albuquerque, New Mexico, where he discovered the life and culture of the Navajo and Hopi Indians in the Great Reservation at Four Corners, covering part of four states in the Southwest of the United States. Hillerman's very original idea was to place his detective stories within the Great Reservation and make his two heroes policemen of the Navajo Tribal Police: a lieutenant near retirement and later retired, and a young recruit, then sergeant, who became his student in the profession. Hillerman, clear headed, is not someone whom humanity drives to despair, because, although not ideal, man is peaceable, because the traditional Navajo interests him. There is also the setting (and archeology) of this region of great natural beauty, its skies, its colors, its reliefs, and contours. As for the riddles and detective investigations that the author recounts, they are well put together and sufficiently captivating to fascinate the reader.

In 1998, Tony Hillerman published new adventures of Lieutenant Joe Lephorn, pulled out of retirement, and Sergeant Jim Chee, officiating as temporary lieutenant, under the title *The First Eagle*. Two unrelated people have disappeared in the wild: a rank-and-file policeman on patrol and a young woman who is a technician and researcher for the Arizona Public Health Department, who studies small mammal vectors (here a colony of prairie dogs and families of kangaroo rats) and various parasite insects (fleas), parasites that are suspected of living and transmitting microbes of infectious diseases. Several human cases of a species of new bubonic plague have shown up on the Reservation. There is indeed an assassin, a young, ambitious researcher working alone for a pharmaceutical company. There is no doubt that Hillerman was inspired by the story of an epidemic brought on by a new Hantavirus, with victims in several states during the summer of 1993, the first of which, setting off the alarm, were in fact Navaho Indians. The virus, at first baptized with the true toponym, *Muerto Canyon*, where the presumed index case was infected, was renamed as *Sin Nombre (!) (engl. = No name)*, so as not to offend the people of the regions and not frighten the public more. We know that the field research for the virus in New Mexico and Arizona was the first great joint maneuver with CDC teams and other concerned organizations—a success since the puzzle was solved and the virus described in just a few weeks. Hillerman's novel succeeds in making us live the daily events of the researchers in the field and in their laboratories, these hunters of viruses and other infectious agents, original players in microbiology research. The author has collected his documentation and information from the best sources.

(pp. 95–96) Well, first he asked whether we'd wrapped up the work on the plague cases. I said no, we didn't know where the

last one got it. I told him Cathy was still working on that one. Then he asked if we'd found any live kangaroo rats up around the Disbah place. That's one of the places where a hantavirus case had turned up. I told him we hadn't. [57]

(p. 102) (. . .) He's studying how some hosts of vectors – like prairie dogs, or field mice, and so forth – can be infected by bacteria or viruses and stay alive while others of the same species are killed. For example, plague comes along and wipes out about a billion rodents, and you've got empty burrows, nothing but bones for a hundred miles. But here and there you find a colony still alive. They carry it, but it didn't kill them. They're sort of reservoir colonies. They breed, renew the rodent population, and then the plague spreads again. Probably from them, too. But nobody really knows for sure how it works.

(p. 115) (. . .) He thinks the bacteria are going to eliminate mammals unless we do something about it. And if they don't get us, the viruses will. He feels this need to warn everybody about it. Jeremiah complex.

(pp. 150–151) "Now, if I'm lucky, the blood in these fleas is laced with *Yersinia pestis* and" – Woody poked the prairie dog with the tweezers-"so is the blood of our friend here. And if I'm very lucky, it will be *Yersinia X*, the new, modified, recently evolved fast-acting stuff that kills mammals much quicker than the old stuff."

(p. 154) "Because nothing stops them except your immune system. You don't cure a viral sickness. You try to prevent it with a vaccine. That's to prepare your immune system to deal with it if it shows up."

(p. 190) "And what you learn from the rodent immune system applies to the human system."

"That's been the basis of medical research for generations," Woody said. He put down his cup. "If it doesn't work this time, we can quit worrying about global warming, asteroids on collision courses, nuclear war, all those minor threats. The tiny little beasties have neutralized our defenses. They'll get us first.

Toward the beginning of his novel, Tony Hillerman has one of his characters tell a historical anecdote, which he ends by citing an age-old refrain still sung today as a nursery rhyme.

(p. 9) "You know," he said, "back in the Middle Ages the doctors had another cure for this stuff. They thought it had something to do with the sense of smell, and they recommended people stave it off by using a lot of perfume and wearing flowers. It didn't stop everybody from dying, but it proved humans have a sense of humor.

(. . .)

"What do you mean?"

They made an ironic song out of it-and it lived on as a nursery rhyme."

Howe sang it in his creaky voice:

Ring around with roses
Pockets full of posies.
Ashes. Ashes.
We all fall down.

The new bacterial or viral dangers have therefore fed and continue to feed the imagination of confirmed or budding writers—our authors above are not the only ones. In addition, a few nonfiction works should be noted, documentaries, written by journalists in the tradition of investigative journalism. We have already cited Laurie Garrett's 1994 *The Coming Plague*. She did it again 6 years later with *Betrayal of Trust: The Collapse of Global Public Health* [37], which emphasizes the errors committed during the last few decades in the research and fight against the microbes of infectious diseases. In 2001, reedited in 2002, Judith Miller, Stephen Engelberg, and William Broad published *Germs, Biological Weapons and America's Secret War* [81], which recalls several minor, but as yet unspoken, acts of bioterrorism, and then underscores the threat of biological warfare, a potential weapon of massive destruction. Finally, from Gina Kolata there is *Flu, The Story of the Great Influenza Pandemic of 1918 and the Search for the Virus that Caused It* [60], whose subtitle explains the content, the first great work on this enormous pandemic that ravaged the planet just at the end of the Great War, a major event in the history of public health that was hidden for a long time. The history of the search for the responsible virus is a novel in itself. In fact, these last books read like novels, with the qualities of American investigative journalism, with its descriptions as lively and colorful as they are precise, and its reconstituted dialogues thanks to the recounts of the people involved in the events, in addition to the documents and archives. This is a form of literature, also artistic in how it presents facts.

September 11, 2001, its aftermath, and the wars in Afghanistan and Iraq, have somewhat discredited the biological threat, especially since it was put forward, along with the chemical and nuclear threats, to justify the political and military actions instigated, the infamous arms of mass destruction now revealed as somewhat overdone. However, the biological risk by voluntary human action still exists. As for more natural biological risk, it continues to thrive, as the lightning SARS episode in 2003 and bird flu demonstrate. There is no doubt that writers and cinematographers will continue to use these risks and the defense against them as subjects for their work. Infectious diseases have a bright future ahead of them in artistic creation.

40.2.7.3 Anticipating a future flu: Hervé Bazin (1911–1996) *The Ninth Day* [4]. Recognized as one of the most talented French novelists, Hervé Bazin devoted his work to the acerbic critique of the human environment he lived in, both in the family, shown in parental intolerance and heaviness, notably coming from the mother, and in the social group and society. He rejected any coercive demonstration of power and showed a discrete benevolence for the rebellious individual or for the unconventional figure. His biting irony toward stupidity and individual or group failings hit home. He was a moralistic writer; his novels were also long fables in prose.

At first sight, one would not have expected Hervé Bazin to launch into writing fiction—or rather science fiction—taking place in an environment describing it, rather removed from his provincial life, or his somewhat reserved Parisian literary life, limiting himself to the Académie Goncourt—and even more so at his age of an octogenarian. But in the end,

the subject treated, although original, was not so far from the intellectual preoccupations of this novelist. The severe and distressing irony of a stupid laboratory accident in a research institute studying fatal viruses could only be interesting to him. In fact, we know that he was informed of the scientific developments in this domain, and of the possible risks, by a close relation by marriage, himself a virologist and researcher—the documentation is complete and faultless. Il existe de ces rencontres entre un bon romancier et un sujet qu'il choisit. L'auteur se doutait que sa carrière ne se prolongerait pas des années, même s'il ignorait que ce serait son dernier roman—not a very optimistic testament, in the end well within the line of his work.

The Ninth Day tells the following story: In a research center on dangerous viruses, financed by the European Community, near the Franco-Belgian border, a program directed by a Doctor Alleaume studying the virus of the 1918–1919 flu, newly found. The organization and the research work are described, as is the private life of the main researchers. A flu from an apparently new virus breaks out in India, in Bombay. The illness is immediately extremely lethal and spreads throughout the world in a few weeks. The political leaders mean to be reassuring, but the economic consequences are tremendous and the panic real: It will take months to produce a vaccine. This flu kills indiscriminately, like all great plagues. We realize that the virus is similar, perhaps identical, to the 1918 virus. The Center is working at less than capacity, affected as everyone is by the number of ill and dead. But it is Alleaume's team that describes the virus first, which allows them to launch the production of an adequate vaccine: They are going to save the world! Well, humanity. The end of the novel can be revealed here: Alleaume begins to have doubts. His main assistant on the project, who has disappeared, had decided to take a break, a few days before the beginning of the flu, giving the impression that he was going to see his ex-wife and children in Canada. But Alleaume learns that Martin Lansdale is spinning out love's sweet dream with a woman and that he went somewhere else. Then comes the official news of his death in Bombay, where he had been staying a few months before, in a hotel, that Alleaume phones: Yes, he was stricken by the flu, among the very first, with the woman, and they had died in the hospital. Alleaume, thunderstruck, then remembers that just before leaving, Landsdale, working on the 1918 virus in the P.4 laboratory, had hit a corner and cracked the mask of his high-security cleanroom suit; but he had immediately followed the protective procedures. But . . . There is no doubt: It is indeed him; they are the ones responsible for the pandemic of the superflu. He imagines the headlines in the newspaper about him: *The rescuer is the assassin!* The next day, a routine exam informs Alleaume that he has advanced liver cancer and has only a few weeks to live.

This novel, with its fatal irony, was the last message from Hervé Bazin to a society that raised his ire. However, as the author specifies, it is science fiction. Like those written by, among others, a certain Jules Verne, we might add . . .

(p. 30) (. . .) Shrewdly built in open country by architects who remembered the Sverdlovsk, alias Ekaterinburg, disaster, where the Romanovs had died and where a Soviet military laboratory had exploded, causing a slaughter, we could see the quadrilateral of the Center.

(p. 38) (. . .) Millesond (the Center's director) was insistent, singing the litany of the sorcer's apprentice:

"And if it was nothing more than that! Accidents, blunders! I can cite dozens. I'll mention just a few random ones, shall I? You can put names on them if you wish. Which good scientist released two rabbits into the wild that he had inoculated with myxomatosis, simply to protect crops? What did a British biologist working at the Porton Research Center die of? And what happened to that poor assistant of some big CEO who was working on the Lhassa virus? Who introduced a macaque virus into a common human intestinal virus, without a single worry as to what would happen if it came back home? Who toyed with the growth hormone gene to obtain rabbits that are bigger than goats, sheep the size of cattle, I mean, monsters? What madman dared wonder if such a thing as ethnic viruses existed that would only contaminate one variety of the population, and for what purpose? (. . .)

(p. 44) (. . .) It's true, that's my role. I must prevent researchers from researching in dangerous areas. I'm not crying mad scientist here; I'm calling out to the virtuous scientist wandering around in the unknown without disclosing that it's a mine field. (. . .)

"Lord! You created the world in six days and on the seventh you rested. On the eighth, you chased man out of earthly heaven to punish him for having touched the fruits of the tree of knowledge. But the ninth day, having gotten a taste of it, *sapiens* gave himself the means to destroy or transform your creation, alone. And he does what he can without necessarily knowing what he's doing. And you see me, Lord, divided between Well done! and Darn!

(pp. 44–45) (. . .) Because, my children, I wish you a good one, like poor Damocles. Live with the very costly nuclear power until the end of time, without ever touching that little bomb, now that would be tricky. But live peacefully, indefinitely, with biologists who exalt in knowing that nature uses the same chemical language for all living creatures, with interchangeable genes, who from now on can break down the barriers separating species and tinker around with ours, who, because of an influx of knowledge, inaccurately measure the immensity of their ignorance, who will provide you with the means to turn life upside down and tease death, all at a low price. Do you really think that's a return to Eden? When a danger is possible, time makes it probable and over the long term, inevitable: it's the story of 0.001 multiplied by 1,000!

(p. 47) "Let's get down to the facts. Since last night, 11 pm, India has been sounding alarms. The flu epidemic that has just started up in the Bombay region can already be regarded as worrying. It seems to be a severe form and the first serological data make us hesitate between a quite dangerous mutant and an old killer reactivated in populations that are no longer immune to it (. . .) It's not at all improbable that it could rapidly spread." (. . .)

Spread to here, of course. To here, where this year's vaccine cocktail distributed to the high-risk members of the population has just been allocated and where people are counting on protection that is now illusory.

(p. 66)) (. . .) Nothing but the virus can do was well as what man has done.

(p. 68) (. . .) the epidemic proceeded like a tumor through distant metastases where the airlines played the role of the blood circulation.

(p. 72) "Imagine, Alleaume, that it's been twenty years ago now that my dissertation director announced the end of the great viral invasions. And now two of them, one right after the other, have declared victory, and three or four more are threatening. The next one could be the resurrection of the yellow fever. In Louisiana, for example." (. . .)

(p. 114) (. . .) The appearance of such a viciously contagious flu the likes of which we hadn't seen since the beginning of the century, even more serious because AIDS has remained undefeated, because dengue fever from the tropics, until now inoffensive, was becoming fatal, because polio was making a comeback, because the planetary upheaval ran the risk of disseminating exotic aggressors that had until now remained local, such as the horrific Ebola from Zaire, the Japanese Kawasaki, the Hantavirus from Korea, the Marburg, Lhassa, Machupo, and Junin viruses and other, less well-known assassins. What? Haven't these attacks been periodical? Haven't they always been responsible for sixty percent of the infectious diseases, these miniscule threads, cubes, rods, spikes, invisible, insensitive, which attack us just like pork, tobacco, potatoes, or cats?

(p. 158) (. . .) But even more significant, there was widespread use of the nose-guard, a small double filter inserted into the nostrils that was supposed to protect the breathing in a contaminated atmosphere and clearly the heir – although made in China – of the perfumed antiplague beaks of the Middle Ages.

(p. 190) In fact, the unfortunate editors recited a single litany: the stock market at its lowest, zero dividends, ineffective advertising, liabilities at their highest, currencies surfing on the money printer, crazed exchange rates, seizures of goods, the IRS with no money coming in, drained budgets. . .A disaster!

(p. 191) With so many apartments empty in the cities, the price per square foot collapsed.

The same can be said of gold, oil, artworks, vintage wines, paintings, art books.

(p. 211) (. . .) Unfortunately, my memory was too good. I hadn't forgotten that the day before his departure, Martin, slipping accidentally, had fallen and struck the edge of the dissection table, that he had cracked the plastic facepiece, and holding his breath – or thinking he was – he had to quickly move back to the security area. Forgotten at the time, this incident later became of vital importance: we would find it in the report.

(p. 215) (. . .) It spread in a circle. The virus itself is showing us: despite its age, it has undergone no change, not even minimal. It surprised me to no end: it was an exact copy of the porcine virus found frozen in Alaska that we reactivated.

(p. 217–218) Am I showing off? Yes, I'm showing off. What did you think I was doing? That in the hundred thousand years to come – small beans, really – not to mention the little bomb, the manipulations would stop, the possible would cease to be solicited, that nature would no longer be more and more rigged by the braggarts of the heroic original sin. What did you imagine? That once smallpox was eradicated (we'll see), AIDS quelled (we'll see), the viral army would back away endlessly? My eye! Death is inventive; so many of its other friends are awaiting their turn. The latest news: see the devourer of human flesh join the list, the bacterium of a violent gangrene, not at all unknown, but rather extolled, that our antibiotics have kindly selected, insistently pushed forward. What did you imagine? *Evil is nothing more that the pus of good*. And our great benefactors, the Jenners and the Pasteurs, like their excellent successors, have so congested the square mile that the *sapiens*, on their own planet, is the first calamity, the source of every other disaster. Let us wish him a good one! After all, when the very saint plague, former world record-holder, caused so many souls to rise to heaven, it never surpassed a success rate of ninety-nine percent! For the superflu, let us come to an agreement, Lord! Your clemency allowed me to remain far off target.

(p. 231) I'm driving along. Custom-fit for me, my cleanroom suit will be of use to no one; perhaps, if the High Security Laboratory is closed, it will become a rare object in the Museum of Fear, like in Japan. I'm driving. The embankments are covered in daisies, with poppies bleeding among them. I too, at my funeral, will be covered in flowers, at the risk of being covered in trash the week after. So what? I will never be aware of it. Everything is fine in the worst case: early retirement, entirely justified, nothing suspicious, unequivocal. I'm putting an end to myself, I'm shutting up, I'm extinguishing myself discretely in the rare satisfaction of disappearing. Salvation, at times, has unexpected appearances.

This novel, like the detective or science fiction works cited before it, is indeed realistic science fiction; it does not recount a story from the past, but perhaps of an approaching future. Any comment would be superfluous.

40.3 CINEMATOGRAPHIC ART: MOVIES AND DISEASES

More than 200,000 movies, including 50,000 movies of the silent screen, have been registered to date [87]. We have explored roughly 11,000 productions (from 1920 to the 2004 Cannes Festival) to identify the implication of infectious diseases in their scenarios. These analyses excluded most documentaries and scientific film production.

Although television films represent an enormous production of movies especially produced for commercial television, we did not include this type of production in our presentation.

Both in the way it is constructed and in which it is received, film is a remarkable art form, allowing as it does the animation of photographic images and the visualization of the temporal development of what is shown. Few arts or art forms, apart from painting occasionally, photography, and sometimes opera, and then in very few works, achieve the evocative (more often brutal than peaceful) passion of film. Film forcibly creates a distortion of reality and time. It makes up for time lapses through flashbacks. It is rare for the duration of a short film, about two hours, without flashbacks, without a return to the past, to be the duration of the story it tells. We are aware of only one real, successful example: *High Noon*, by Fred Zinnerman (1952), which obeys, in a real, albeit psychological western, classical drama's three unities: unity of action, place, and time.

Moreover, it is good form to create an analogy with literature and speak of film writing. Despite the good impression this makes, film is nothing like literature. Of course, there is a screenplay written by one or many authors, which is divided up into scenes (shots and sequences), a script which describes the action and the characters' dialogues (today a storyboard is drawn, most probably by computer); next comes the direction and the shooting; finally, an essential part: editing, which plays a critical role in the success or failure of a film. Hence, we should use the term "film language" rather than writing. On top of that, film is a collective artistic work of a team made up of many members, even though celebrity is often reserved only for the directors, screenwriters, and actors—there is a project manager, sometimes more than one, just as in architecture. A writer, however, is alone with his page and his pen, or in front of his computer with his keyboard. In order to present a narrative or tell a story, everything in the spirit of the work and in its very construction is different. However, the main difference between the two narrative (and in a sense spoken) arts—one ancient and one modern—resides elsewhere. The narrative is received by the reader in a physical, physiological, and spiritual way, on the one hand, and the cinemagoer on the other hand. By definition, a literary work is read, but a work on screen is watched and also listened to, both senses being almost the only ones brought into play. Instantly, the brain interprets the work as it wishes and delivers sensations and emotions. The cinemagoer (as is the case with a play or an opera) can be illiterate. The reader of a work of literature must of course know how to read—the first intellectual operation—not only how to decipher letters and words in a sentence and make sense of its syntax but he must also understand and reconstruct what is described: the second intellectual operation. The space left up to the reader's imagination is vaster and more intimate and discreet than that left up to a viewer. The latter receives the message and the image frontally; he is obliged to absorb it without being able to analyze it as the film progresses. That is why, for example, a violent, crudely brutal and bloody act is felt with much more sensitivity in a film than one described in a literary text. The same goes for sex, which on film is always arousing through its visual aspect, which often creates unease—un-ease/dis-ease—like disease. In literature, the acts described have to be outrageously perverse and sadistic, with in-depth details to create similar unease in the reader: Only a few decades ago, this type of narrative was censored and condemned, the authors and publishers prosecuted. Even today, when freedom of speech has asserted itself at least in Western cultures, explicitly cruel and/or sexual narrative is not freely accessible to the average citizen (it is restricted to pornographic magazines, that is, photography, and Japanese mangas, or comic books, as well as some other media or vectors). However, film is readily accessible. From the very start, film has been aggressive. It has become more so, through freedom of speech but also thanks to technical progress.

The general comments above on the art of film do not form an opinion, but the acknowledgment of a reality, without criticism or value judgment. Why have we emphasized the technical reality of film? Why have we brought it up here? What does it have to do with our subject? Doubtless not everything, but a great deal. Indeed, suffering, death, biological and/or mental ill health should be main themes in film. Indeed they are, but not much through the means of disease, and certainly not through contagious or epidemic disease. Nevertheless, the situations created by such diseases in reality, in real life, should lend themselves to the film medium. We note, however, that film has only provided a small number of works concerning infectious diseases and epidemics, to a greater or lesser extent: Out of 11,000 films listed in a dictionary by specialists, fewer than 100 are about disease or evoke the subject.

Note that literature, on this level, is hardly rich in number, even counting the recent and contemporary thrillers inspired by emerging diseases and microbes. On the contrary, ordinary suffering, violence, cruelty, and sadism—of war for example—are widely treated, but rarely through disease. Disease, a fairly natural biological phenomenon, creates unease, and when it is potentially fatal, creates fear. We can see no other major explanation for its absence noted in the literary arts. Filmmakers have used infectious disease, either by adapting a work of literary fiction, or by taking inspiration from reality, or by inventing an original story. Let us take a look at the main works featured in our table.

To recap: 87 films out of 11,000 quoted in the reference book is a small percentage. Almost all 87 titles are works of quality; some of them are masterpieces. Their directors are often filmmakers who are well known for the quality of their work, including such living artists as Bergman, von Trier, Wadja, Russel, Mulligan, Carpenter, Cronenberg, Demme, Peterson, Herzog, Robson, Coppola, Branagh, and Gillam. Those who have died include Manzies, Brow, Kazan, Preminger, Demy, Vidor, Cukor, Polack, Bresson, Huston, Wyler, Renoir, Allegret, Ford, Grémillon, Reed, Murnau, and Visconti. Almost none of the others were or are producers of simple popular entertainment consumer products, which are also part of film; they were or are skilled craftsmen.

The oldest film made directly concerning our subject is entitled: *Au Ravissement des Dames*, directed by a certain Alfred Machin, dating from 1913. It is a short film that lasts only 11 min. (Only short films were made at this pioneering era of the cinema, with either a dramatic or a comical subject, with accentuated visual effects due to the lack of sound.) Its content is, however, original and curious: the dressmakers of a department store are exploited and live in miserable conditions. They become infected with tuberculosis and contaminate the rich clients with the dresses as vectors. This is one of the first social commentary films, if not the very first. Note that nearly 100 years later, at the beginning of the twenty-first century, disease associated with working conditions and the working environment is again in the news.

The Plague makes its first appearance in film with Otto Rippert's *Die Pest in Florenz*, in 1919, just after the end of the First World War, in the heat of the Spanish flu epidemic, perhaps not a simple coincidence.

TABLE 40.1. Filmography of Infectious Diseases

Movie Title	Author	Origin	Date	Remarks (the Place of the Disease in the Scenario)*
Plague				
Die Pest in Florenz	Otto Rippert	Germany	1919	Referenced to the 1348 black plague of Florence? (1)
The Painted Veil	Richard Boleslawski	USA	1934	Fighting epidemic in China ? (4)
The Seventh Sin	Ronald Neame	USA	1957	Remake of *The Painted Veil* (4)
Things to Come	William C. Manzies	United Kingdom	1936	From HG Wells, war, plague, revolt, anticipation (1)
The Rains Come	Clarence Brow	USA	1939	Monsoon, plague and nurse (3)
Forever amber	Otto Preminger	USA	1947	Plague epidemic in the 17th century in kingdom of England
Monsieur Vincent	Maurice Cloche	France	1947	St. Vincent de Paul
The Rains of Ranchpur	Jean Negulesco	USA	1955	Remake of *The Rains Come* (3)
Panic in the Streets	Elia Kazan	USA	1950	Risk of disease spreading (1)
Det Sjunde Inseglet	Ingmar Bergman	Sweden	1956	Epidemic and death dance (1)
Andrej Rublew	Andrei Tarkowski	URSS	1969	Russian plague in 1424 (1)
The Last Valley	James Clavell	USA	1970	30-years war, plague episode (2)
The Pied Piper	Jacques Demy	United Kingdom	1971	The Pied Piper tale (1)
Flesh and Blood	Paul Verhoven	USA	1985	16th century war and plague in Europe (4)
Epidemic	Lars Von Trier	Denmark	1987	Plague Milano 17th century (2)
Niezwskla Podroz Balthazara Kobera	Wojcieck J. Has	Poland / France	1988	Plague and societies, 16th century (4)
Tuberculosis				
Au Ravissement des Dames	Alfred Machin	France	1913	Plague and societies (1)
La Bohème	King Vidor	USA	1926	From Puccini opera booklet written by Henry Murger (3)
Mimi	Paul Stein	USA	1935	Remake of *La bohème* USA (3)
Camille	George Cukor	USA	1936	From Marguerite Gautier novel (3)
Docteur Laennec	Maurice Cloche	France	1949	Biography (2, 3)
La Dame aux Camélias	Raymond Bernard	France	1953	From Dumas novel (3)
Traviata	Vittorio Cottafavi	Italy/France	1953	From Dumas novel in modern times (3)
This Property is Condamned	Sydney Pollack	USA	1966	From Tennessee Williams (3)
Mouchette	Robert Bresson	France	1967	The mother of heroin (3)
Brzezina	Andrzej Wajda	Poland	1970	Psychological drama (2)
Priest of Love	Christopher Miles	United Kingdom	1981	A biography of the last years of DH Lawrence's life (3)
La Bohème	Luigi Comencini	France/Italy	1987	From Puccini opera booklet (3)
Cholera				
The Barbarian and the Geisha	John Huston	USA	1958	Epidemic in Japan in the 19th century (1)
The Charge of the Light Brigade	Tony Richardson	United Kingdom	1968	War and diseases Crimea 1854. A 1936 movie remake of M. Curtiz, USA (4)
The Music Lovers	Ken Russel	United Kingdom	1968	Psychological drama (2)
Le Hussard sur le Toit	Jean-Paul Rappeneau	France	1995	An epidemic in South of France in 1030 (1)
Leprosis				
L'Homme du Niger	Jacques de Baroncelli	France	1939	Colonial Adventure (3)
Ben Hur	William Wyler	USA	1959	A remake of 1925 silent movie by Fred Niblo, USA (4)
The Spiral Road	Robert Mulligan	USA	1962	Borneo epidemic in 1936 (2)
The Fog	John Carpenter	USA	1979	Fantastic, horror (2)
Smallpox				
Gösta Berling Saga	Mauritz Stiller	Sweden	1924	Middle Ages drama, adapted from Selma Lagarlöf (Nobel price) (4)
Nana	Dorothy Arzner	USA	1924	From Emile Zola (3)

TABLE 40.1. *(Continued)*

Movie Title	Author	Origin	Date	Remarks (the Place of the Disease in the Scenario)*
Nana	Jean Renoir	France	1926	From Emile Zola (3)
Nana	Christian Jacques	France/Italy	1955	From Emile Zola (3)
Les Liaisons Dangereuses	Roger Vadim	France	1960	Drama; From P. Choderlos de Laclos (3)
Dangerous Liaisons	Stephen Frears	United Kingdom	1988	Drama; From P. Choderlos de Laclos (3)
Valmont	Milos Forman	USA	1989	A remake of *Dangerous Liaisons* (3)
Typhus				
In Old California	William NcGann	USA	1942	Western typhoid epidemic (2)
Les Orgueilleux	Yves Allégret	France/Mexico	1953	Phycological drama in Mexico (2)
Die Letze Brück	Helmuth Kautner	Germany, RFA	1954	War and murine typhus (2)
Morte a Venezia	Luchino Visconti	Italy/France	1971	Cholera or typhus? (2)
Yellow Fever				
The Prisoner of Shark Island	John Ford	USA	1936	Historical drama (Lincoln murderer, doctor) (2)
Jezebel	William Wyler	USA	1938	Drama, New Orleans quarantine (2)
Rabies				
Gardiens de Phare	Jean Grémillon	France	1929	Drama (1)
The story of Louis Pasteur	William Dieterle	USA	1936	Biography (2)
Rabid	David Cronenberg	Canada	1976	Horror: vampire and rabies (2)
Syphilis				
Liebes Konzil	Werner Schroeter	Germany	1981	Fantastic (1)
Le Mal d'Amour	Giorgio Treves	France/Italy	1986	Drama (1)
AIDS				
Encore (Ones More)	Paul Vecchiali	France	1988	Drama (1)
Les Nuits Fauves	Cyril Collard	France	1992	Drama. The main actor succumbs to AIDS shortly after (3)
Philadelphia	Jonathan Demme	USA	1993	Drama (1)
Fever and hemorrhagic fever				
Outbreak	Wolfgang Petersen	USA	1995	Adventure (1)
Aguirre, der Zorn Gottes	Werner Herzog	Germany	1972	Historical adventure (4)
Ridicule	Patrice Lecomte	France	1996	Comedy; malaria, and politics (4)
Vaccine, therapy, and politics				
Arrowsmith	John Ford	USA	1931	Epidemic, vaccine; from Sinclar Lewis story (1)
Ashanti	Richard Fleisher	USA/Switzerland	1978	Vaccine (2)
The Third Man	Carol Reed	Great Britain	1949	Antibiotic smuggling (4)
The Constant Gardener	Fernando Meirelles	Great Britain	2006	Phase II to III Drug development = inocuity and efficacy (1)
Bioterrorism related				
The Satan Bug	John Sturges	USA	1965	Bacteriological war (1)
Cassandra Crossing	George Pan Cosmatos	USA	1977	Bacteriological war: Terror in a train (1)
Avalanche Express	Mark Robson	USA	1978	Bacteriological war (2)
Mauvais Sang	Léo Carax	France	1986	Virus robber (2)
Mission Impossible II	John Woo	USA	2000	Virus, vaccine and spy (4)

Legend:

1. The disease has a central role in the scenario; the disease plays a major component within the plot.
2. The disease appears clearly mentioned as a component of the plot but does not overwhelm the entire scenario.
3. The disease is responsible for the death of the main(s) character(s).
4. The disease is secondary and more a part of the decor than the plot. It is part of the environment as a background.

The four other films of the silent era relating to infectious diseases are high-quality films, if not masterpieces, beginning with Murnau's *Nosferatu* in 1922 (see the commentary below on vampirism). In 1926, King Vidor adapted *La Bohème*, a novel and opera of the nineteenth century, and Jean Renoir adapted *Nana*, based on a Zola novel, the first film creations on the theme of the woman in love and/or prostitute, who dies in her youth and beauty of tuberculosis, or, when the character is a perverse exploiter of men, of smallpox. Finally, in 1929, Jean Grémillon produced *Gardiens de Phare*, a tragedy set off by rabies: A young man is bitten by a rabid dog; he joins his father who, like him, is a lighthouse keeper. The disease breaks out; there is a fight in the lighthouse, and the father ends up sending his son down into the stormy sea. This film was one of the precursors of modern horror films, but the quality of the direction (the scriptwriter was Jacques Feyder, another great filmmaker), its sobriety, the sets, including in the outdoor scenes, made for a first-rate dramatic work, showing the public the effects of one the most dreadful infectious diseases, which, once it breaks out, leads inevitably to a ghastly death, with no chance of remission.

Sound and voice in cinema. Let us immediately award our Palme d'or, both generally and within our theme: Ingmar Bergman's *The Seventh Seal* (1956), which takes place in an indefinite period, toward the end of the Middle Ages, during a plague epidemic or even the Great Plague. A knight and his squire, returning from the crusades to their country, which they discover ravaged by the plague, in a state of total moral and social torment. The knight goes to meet Death, suspended between despair and hope. The filmmaker proposes a series of scenes from accounts and images of the plague: misery, famine, terror, the cadaver carts, the processions of flagellates, witches burned alive, and so forth. Finally, the knight arrives at his castle at the head of a death dance. This film is a metaphysical quest, and, as one critic has written: [it] "never ceases to pester—in the positive sense of the word—the viewers' intelligence and sensitivity" [87]. Other films take place during an episode of plague from centuries past (e.g., *Andrej Rublew*, *Niezwslka Podroz Balhtazara Kobera, and Epidemic*), but more as a dropback than as the central subject, without achieving the realist and poetic grandeur of *The Seventh Seal*. We should also mention *The Pied Piper*, by Jacques Demy, 1971, an adaptation of a German tale staging a mysterious pied piper of the fourteenth century, who, in the city of Hamelin, attracted all the rats that were carriers of the plague by his music, leading them to the river where they drowned. However, because the town's population did not reward him for his act, he leads away all the town's children, who follow his music not to the river but to the Land of Songs (in the tale on which the film is based, the end is even more cruel, with the piper drowning the children).

Epidemics. A subtheme often comes back in films that could be qualified as epidemics: a character's heroism, whether it be a man or a woman, doctor or nurse, who sometimes dies as a result, with the action often taking place in an exotic setting. For example, *The Rains Come*, and its remake *The Rain of Ranchpur*, adapted from the Louis Broomfield novel, which takes place in the time of the British Empire in India, with the plague interfering with the characters' love affairs: *Forever Amber*, in eighteenth century London, the story of a courtier; *The Barbarian and the Geisha*, in nineteenth century Japan; *The Spiral Road*, in Borneo; *Die Ketze Brück*, with a German woman military doctor, prisoner of the Serbian partisans and caring for typhus victims; *Ashanti*, with a vaccination campaign deep in Africa; *Arrowsmith*, where a doctor sacrifices his life for his profession; *In Old California*, with a typhoid epidemic in the Sacramento of nineteenth century California; *The Painted Veil*, and its remake *The Seven Sins*, where a Western couple combat a plague epidemic in China.

John Ford's *The Prisoner of Shark Island*, 1936, was based on a true story: a doctor cares for President Lincoln's assassin without knowing it. Arrested, he is sentenced to hard labor for life on an island off the coast of Florida, in an unhealthy climate. A yellow fever epidemic breaks out, and the doctor sacrifices himself to care for the victims. His attitude gains him a pardon, his wife fighting for the pardon in Washington. This film on heroism and duty is treated voluntarily by its director on a grave and austere tone.

Yellow Fever plays a role in another film, *Jezebel*, by William Wyler, 1938. It takes place in 1850 in New Orleans: At a ball, a young and rich heir, temperamental, provokes her fiancé, who leaves her and marries another woman. After a period where she continues to behave in an unpleasant and excessive manner and is the cause of various dramatic events, she learns that her ex-fiancé has yellow fever. She joins him in the lazaretto where he is confined, promising to marry him in redemption and to change her ways. Bette Davis's personality suits this character perfectly, and she wins an Oscar for her role in this classic film. Infectious disease and its threat of death push the young woman, frivolous and perverse, to sacrifice. This type of female character is like a model, even if it has been presented in a variety of ways in its amorous seduction: many films were adapted or inspired by this model such as *Nana*, or *Les Liaisons Dangereuses*, or even *La Bohème*, and of course *La Dame aux Camélias*. George Cuker's *Camille*, 1936, is undoubtedly the best film inspired by the story of Marie Duplessis, with Greta Garbo at the summit of her glory in the starring role. Infectious disease, tuberculosis, or smallpox dramatize the divers romances or romantic adventures in these films.

Les Orgueilleux, Yves Allégret, 1953, is a remarkable film on more than one account. In a small, desolate Mexican port, where there has been an epidemic of meningitis, two Frenchmen meet: a young woman, adrift, and a former doctor, responsible for the death of his wife and alcoholic. One scene in particular has become famous, a sort of medical eroticism, as it were, where the doctor (Gérard Philippe) performs a spinal tap on the woman (Michèle Morgan), in a light gown that reveals more than it covers. The dramatic intensity of this film is exceptional; but love will revive hope.

In a slightly different spirit, a detective film is interesting: *Panic in the Streets*, by Elia Kazan, 1950. The action takes place

in New Orleans, where an illegal immigrant has been murdered in the port, but the victim is discovered to be a carrier of the plague. A doctor has 48 hours to find the murderers and prevent the infection from spreading. The suspense is handled well, and this film is partly documentary, filmed in a natural setting, underscoring the strangeness of this very particular American city.

This Property is Condemned, Sydney Pollack, 1966, sends us to the same city (and the Mississippi valley), but the story takes place in 1930, and the subject is quite sordid and even darker. A teenage girl falls in love with a drifter, to her mother's great displeasure. He leaves and in spite the girl marries another man. She then flees to the big city to join her first love, who, learning of her marriage, abandons her. She becomes a prostitute and dies of tuberculosis. This is the story of a girl in love but lost, and who dies of her situation, in the America of the Great Depression. The director makes his actress, Nathalie Wood, shine, and succeeds in making this desolating story poetic. Here, disease is both punishment and deliverance.

Death in Venice, Luchino Visconti, 1971, does not require presentation. This film, of an extreme estheticism treating shocking subject, led to great debate and was both admired and disparaged. Those who had read Thomas Mann's novel, from which the film was adapted, may have been disappointed. In the film, the epidemic that reinforced the morbidity of the story is portrayed as typhus; the book uses cholera.

Music Lovers, Ken Russel, 1968. This is a curious musical biography of Tchaikovsky, portraying this composer as having a tormented psyche, with the preposterous idea of inoculating himself against cholera by drinking a glass of contaminated contents, haunted "as he was by the image of his mother dead of the disease when he was young."

Cholera. It took the cinema more than 40 years to take on *The Horseman on the Roof*, directed by Jean-Paul Rappeneau in 1995. The film, the epic that it is, suits cinema well, despite the length of the story and the variety of situations and sets. But if one has enjoyed Giono's novel, this heroic story at the heart of a cholera epidemic, one can only be disappointed by the film (as is often the case of films adapted from great novels). The film emphasizes the love story over the realities of the epidemic and the passions that it releases—entire episodes from the book have been cut. However, the scenes of the measures taken by the police and the time spent in the quarantine station are interesting. Two other great contemporary novels should also be noted: *The Plague* and *Love in the Time of Cholera*, neither of which has yet been made into a film, to our knowledge.

Leprosy. Our table includes *L'Homme du Niger*, directed by Jacques de Baroncelli, 1939, because it is a colonial film, rather rare, and it speaks of leprosy, this long-course disease, silent but visible. In William Wyler's *Ben Hur*, 1959, the relation to this disease is very much in the background: the hero's mother and sister are afflicted, as seen at the end under their veils. But they will be miraculously cured by Jesus.

The Third Man, Carol Reed, 1949 (Palme d'Or in Cannes), a film famous for its music, its postwar images of Vienna occupied by the Allies and how music was pursued in the sewers of the city. This film relates to our subject in that the dark hero, played by Orson Welles, is at the head of antibiotics smuggling (they were still rare at the time), notably against tuberculosis, thus preventing children from being saved.

Films inspired by bioterrorism appeared in the 1960s and 1970s, incredible stories but more or less possible: A violent virus developed in a secret laboratory and stolen (*The Satan Bug*, John Sturges, 1965); a lethal virus, transported by a terrorist, accidentally gets out on a train (*Cassandra Crossing*, G.P. Cosmatos, 1977), with its famous but aging actors; a bacteriological war project during the Cold War (*Avalanche Express*, directed by Mark Robson, 1978); or a stolen virus (*Mauvais Sang*, Léo Carax, 1986); finally, a pharmaceutical company that is ready to use a virus to sell its vaccine that protects against it (*M.I-2*, directed by John Woo, 2000).

We would first like to mention three themes, which have been widely used in the scenario of a number of films, namely the man-made man, Dracula and the vampires, Frankenstein, and the living dead. All of them have a particular relation to infectious disease.

Dracula: As does Frankenstein, the myth of Dracula as a vampire comes from literature: *Dracula* by Brahm Stoke. Dracula and mostly Nosferatu are seen to arrive by boat in London along with the rats and the myth of the Black Death. Moreover, the transmission of vampirism strictly mimics an infectious transmission by bite, with the bitten person becoming infected and becoming a vampire. The vampire appears as a vector of a plague and like the Black Death transmitted to blood.

TABLE 40.2. Vampirism and Vampires

Movie	Author	Country	Year
Nosferatu: Eine Symphonie des Grauens	Friedrich Wilhelm Murnau	Germany	1922
Nosferatu, Phantom der Nacht	Werner Herzog	Germany/France	1979
Dance of the Vampires	Roman Polanski	United Kingdom	1967
Dracula	Ted Browning	USA	1931
Dracula, Prince of Darkness	Terence Fisher	United Kingdom	1966
Dracula	John Badham	USA	1979
Dracula	Francis Ford Coppola	USA	1992
The kiss of the vampire	Don Sharp	United Kingdom	1962
Interview with a vampire	Neil Jordan	USA	1994

TABLE 40.3. Horror and Science Fiction

Movie	Author	Country	Year	Them
The Invasion of the Body Snatchers	Don Siegel	USA	1956	Grains from space invade the Earth (1)
The Mask of the Red Death	Roger Corman	USA	1964	Based on a short story by Edgar Allan Poe (1)
The Plague of the Zombies	John Gilling	Grande Bretagne	1965	Horror: Mysterious epidemic (2)
Parasite Murders	David Cronenberg	Canada	1974	Horror; *Fr.* "Frisson" (1)
Die Hamburger Krankheit	Peter Fleischmann	Germany (RFA)/France	1979	Fantastic drama (1)
Night of the Living Dead	George A. Romero	USA	1978	Horror; Mutation ? (1)
The Crazies	George A. Romero	USA	1974	Horror, Virus (1)
Mary Shelley's *Frankenstein*	Kenneth Branagh	USA	1994	Epidemic (2)
The Omega Man	Boris Segal	USA	1971	Science fiction (1)
The Andromeda Strain	Robert Wise	USA	1971	Science fiction (1)
Twelve Monkeys	Terry Gillam	USA	1995	Science fiction (2)

Frankenstein or the modern Prometheus [92]: Adapted form the novel written by Mary Shelley, several movies of a range of artistic value have been produced. The link to our theme comes when the hero, a doctor who wanted to create a human being, uses body parts from "donors" who had died during a previous short epidemic; the troubled doctor was worried about whether the different parts were fresh and matched in terms of time of death. Table 40.3 lists the two main versions of the *Frankenstein* story: James Wall's film made in 1931, with Boris Karloff, and Kenneth Branagh's, 1995. There is both fantastic and horror in the story written by Mary Shelley, the wife of the famous poet—the hero she imagined wishes to produce an artificial man, a sort of robot, from pieces of several cadavers, but the cadavers are infected and the new being sows terror and spreads an epidemic. It should be noted that there were roughly 20 versions of *Frankenstein*.

The living dead. The third theme related to death appears essentially in the horror film genre, whereas the others reflect on humans and humanity.

Quite similar themes can be found in a series of films that we have listed in the table we call *The living dead*. However, they are often science fiction or horror films. A few titles: *The Plague of the Zombies*, by John Gilling, 1965; *The Night of the Living Dead*, by G.E. Romero, 1974; *Invasion of the Body Snatchers*, Don Siegel, 1956, with a remake in 1978; *The Andromeda Strain*, directed by Robert Wise, 1971, is an adaptation of a novel that made its author, Michael Crichton famous (see Part B); and *Twelve Monkeys*, by Terry Gillam, 1995, passing from bioterrorism to the struggle of an ecological movement, making use of a sort of journey to the past (or to the future!).

In a totally different genre, ***The Fog***, by John Carpenter, 1979, skillfully uses the theme of cursed disease, long considered a punishment for sins committed, in this case leprosy. A ship transporting lepers in the nineteenth century is shipwrecked by the inhabitants of a coastal village. The victims come back 100 years later to take revenge. It is quite a successful Gothic horror film, a genre that has come back into fashion since the beginning of the 1970s. David Cronenberg's *Rabid*, 1976, is typical of the period, where the phantasmagoria mix with terror. In this film, the filmmaker associates vampirism and rabies.

The Mask of the Red Death, Roger Corman, 1964, is taken from one of the Gothic short stories of Edgar Allan Poe (even though the story is short, just a few pages, we have cited parts of it in Part B). The story: The prince Prospero, who made a pact with Satan, gives a great ball, where Red Death (the plague) has invited itself. "This adaptation is a classic of the genre," and therefore deserves to be listed in the table of horror and Satanic films, rather than in the table on the plague.

40.3.1 The Ebola Fever Movies and Television Movies

Ebola fever emerged in 1976 "in the heart of the shadows" in the Democratic Republic of Congo (former Zaire), crossed by the Ebola river. Ebola fever went hidden for 25 years and reemerged in Kikwit, 300 km south of where it first appeared. Jens H. Kuhn (Jens H. Kuhn, pers. Comm. The Filoviruses—History and Research C. Calisher Edit.—Unpublished) gives us an extensive analysis of the scientific and nonscientific production, providing insights on how and why Ebola fever fueled the popular imagination and inspired literature and cinematographic art.

In an authoritative compilation of all production of all kinds, Jens H. Kuhn provides an extensive view of the Filovirus arcane including Ebola fever and Marburg disease, their history, and the consequences on public health and social issues

(. . .)

. . . a substantial interest in the filoviruses has developed among the general public, in part because of novels and popular science stories and Hollywood productions that portrayed them.. . Poets [94] and artists [62] seem to be inspired by the filoviruses; literature analysts suggested that an ebolavirus outbreak might have inspired Edgar Allen Poe to write *The Masque of the Red Death* [102]; and investigators used "Ebola" as a catch phrase to draw attention to their articles, many of which did not pertain to filoviruses.

(. . .)

"A new subgenre of horror movies, termed plague films [104], and television productions amplified the public's concern

(. . .)

It has been established that most of these plague films disregarded scientific facts and focused on the "rhetorically constructed, predatory nature of the [filo]virus" to attract interest [104]. Other analysts came to the conclusion that the same is true for popular scientific articles, which display the filovirus as killers with will [88]. Comparative analyses demonstrated that fictitious work and Hollywood productions portraying filoviruses are construed similarly [90]."

In addition, a great deal of literature has been published on the subject, and it appears from time to time in the news [18] (see also Section 40.2.7).

Outbreak was a huge Hollywood production, produced in 1994–1995 by Wolfgang Petersen, inspired by Richard Preston's novel *The Hot Zone* as soon as it came out. The story imagines the appearance of an Ebola-type virus in the United States, which is reported to have mutated and is now propagated by breathing and the breath. A military plot is woven into this story, because the military does not want to use an existing, but secret, vaccine in response to the epidemic. The story is quite plausible, well documented, and for the first time shows the CDC's specialized civil team in action, launching the chase for the new virus, as well as the Army's research center (USAMRIID: United States Army Mediral Research Institute for Infectious Diseases) at Fort Detrick (Frederick, Maryland). The audience is pulled into the suspense. The two star characters were based on a husband and wife team of real virologists, pioneers in the hunt for viruses of hemorrhagic fevers such as Machupo and Ebola.

40.3.2 Alien "The Series"

"The stranger" is a parasite: It lays eggs; it feeds on its host (human or animal); and it undergoes metamorphoses that include an intense predation phase before becoming adult. Such is the description of the perfect cycle of an arthropod-type parasite. The quest for the host indispensable to the survival of the species takes it into outer space and, like some parasites, sends it into a stage of resistance and/or hibernation when prey becomes rare.

In conclusion to this part on cinema, we will say that this art has produced a wide variety of works even remotely related to infectious diseases or epidemics. Compared to world film production, infectious diseases account for a minor part in scenarios, certainly if we compare it to the themes of war or love, as we will see in conclusion. On the contrary, the theme of infectious diseases has been used remarkably well in this art, often transposed from literature to the screen. Today's health news has played a role and the cycles of humanity's great fears have also been a driving force in this production.

40.4 THE ARTS AND INFECTIOUS DISEASE, IN CONCLUSION

Other artists have taken inspiration from infectious diseases, either directly as did the poets afflicted with disease or indirectly as did architects in their hospital creations. Here are a few examples to conclude this chapter that can never be truly closed, like a work of art, which is never finished.

In architecture the examples are many, from homes not only to protect the well, for hospitals to cure but also to isolate the ill. Throughout the history of medicine, architects have played a role and built on the sites chosen (sanatoria and spas) and in the city, not necessarily within the restrictions of health needs.

Facing Marseille, the Caroline Hospital on the Frioul Islands (1828) can be considered the masterpiece of Michel-Robert Penchaud (1772–1833), architect of the city of Marseille: ". . . a universal lazaretto . . . a place of refuge accessible in time of peace as in time of war, in case of contagion, to sailors from all nations, a religious sanctuary, both civil and political." [13] Since the sixteenth century, with its epidemics, from the plague to yellow fever, the Frioul Islands have been a protective belt for the city.

The Paimio Sanatorium, by the Finnish architect and town planner Alvar Aalto (1933), is a major work that appears on the first page of the *Encyclopédie des arts* [34] and is remarkable for its representation of functionalism expressed by an elevated, fine, and airy architecture, at the service of the patients' well-being and human relations.

Music and dance are intwined, and the dance of death, figuring in the plague epidemics, shows death with a musical instrument, which seeks to seduce, enchant, and attract mortals. This seductive symbolism attributed to music is found in the pied piper of Hamelin, who combats rats (the plague) and then takes revenge on the ingratitude of men and becomes the instrument of Death. Later, this theme of the death dance inspired musicians such as Franz List, Austrian composer who, in 1849, proposed a suite of variations on the theme *Dies irae* "Totentanz" (The Dance of Death) or later, Camille Saint-Saëns, a French pianist and organist, who composed a symphonic poem: "The Death Dance" (1886). From the Middle Ages to our time, "The Death Dance," an inheritance from the great plagues, has been sung.

In poetry disease is also present. Again the plague, with the famous poem by Jean de La Fontaine, *The Animals Stricken with the Plague*. (Verses 1–7) [63].

A blight whose very name gives cause
For fear and trembling;
on that was Invented by the gods
and sent, in fact, As punishment.

Fig. 40.20. Caroline Hospital on the Frioul Islands. The building is registered as a Historical Monument. In 1821, the government ordered the construction of the Berry Dike, joining Pomègues to Ratonneau, and the Caroline Hospital—a homage to Marie-Caroline de Bourbon-Sicile, princess of the Two Sicilies, wife of Duke de Berry—the creation was ordered from the architect M.-R. Penchaud. Used for the great epidemics of the nineteenth century for quarantine, the site was partially destroyed by the Allied bombings during the liberation of Marseille in August 1944. (a) An overall view of the Caroline Hospital. (b) In the background, Marseille. (c) The chapel, located at the center of the hospital complex (Moziconnacci photograph; courtesy of the Association Caroline, which operates the monument).

> The Plague – for why should one Not call by its name? –
> waged war Upon the beasts.
> Each day saw more and more
> Enrich the waters of the Acheron.
> Some lived, but all were touched.

Or leprosy avec Jean Bodel and *Adieux to the Lepers* when, "Toward 1205, he was readying himself to go on a crusade he was inflicted with leprosy. (. . .) In his *Congé* (he understands *Adieux to the Lepers* as announcing Villon's *Testament*), about to be locked up in a leperhouse, he commends himself to the prayers of his friends and his Arras protectors" [31]

> *(. . .)*
> *The pain descending into my heart*
> *With your face ravaged and pale*
> *and who makes me meek,*
> *before I pack my case, (. . .)*
> *I leave ill and crippled*

Comics are a part of these arts that has emerged with the technologies of the last century (the airbrush technique and computer-assisted drawing). The master ideas come from classical literature and borrow from the bacteriological threat. Thus, *The Voronov Affair* by Yves Sentes and André Juillard [91] takes place during the Cold War when a Soviet rocket, hit by a meteorite, falls to earth with a bacterial spore attached to it. Identified as the new "Z bacterium," its discoverer attempts to use it as a biological weapon. Children's thymus protects them against the infection and makes them healthy but infecting carriers via airborne transmission. They are the living vectors used by Professor Voronov to get rid of the leaders of the world and take power. The idea is modern—terrorism and bacteriological weapons—the scenario is well constructed around the investigations of the British monarch's secret service, MI5, and heroes are well known to an audience of impassioned enthusiasts. Precise drawings and meticulous coloring have made the reputation of this collection created by J. Jacob.

For a younger comic book audience, but just as celebrated, the authors Tome and Janry propose *Virus* with its heroes Spirou and Fantasio, who must protect the world from a lethal virus that has escaped from a secret laboratory isolated in the polar ice [96].

Thus, all forms of art have treated and continue to treat infectious disease, today's and yesterday's literature, with cinematography today providing the most voluminous production. Although the great themes that have inspired artists were often love, war, and religion, disease, and in particular infectious disease, has its place in the decor of this focus, and it provides the artist with matter for inspiration. Fruit of love, it is the diseased child who magnifies this unique sentiment and the need for compassion. The pandemics that ravaged towns and villages are a reflection of the warring hordes that helpless people regarded with terror and resignation: Power and suffering come together. The gods are present in popular imagery; they govern the plagues and use them to punish humans and their faults.

Like the great fears that occupy the collective unconscious, infectious diseases, through their message of death and their epidemic genius, serve artistic creation: It is up to

Fig. 40.21. Virus.

the artist to make use of infectious disease to show, guided by the scientist, what the eye cannot see of the life of microbes.

We always come back to Fear, Death, to the Fear of Death. Wars, famines, and plagues—the assassinating and raping war, hunger that can push man over the edge to cannibalism (Géricault's painting: *Raft of the Medusa,* where the dying are represented in the same postures as the plague victims of Marseille a century earlier), and disease often insidious and sometimes brutal. The Apocalypse adds the wild beasts that frightened (The Middle Ages invented monstrous and hybrid imaginary beasts); animals that certain peoples captured to have them massacre and devour human beings, for the pleasure of crowds of spectators.

Through infectious diseases and epidemics, Death ravages, and men moan like women and also cry "why?," the eternal question of *Homo sapiens sapiens* for at least 100,000 years – the oldest prehistoric burial ritual known. Despite all the woes, all the misery of this world and among its beauties, humans are still here: six billion two hundred million we are told, and the numbers increase every day, with the same suffering, the same fears, and the certainty of death. There is, however, the fabulous progress of medicine, but beware of the demiurges and those who consider themselves God, Mary Shelley told us, 124 years ago.

So let us cite one last time an artist, novelist, and poet, Blaise Cendrars, who wrote in 1913, one year before the First World War, in his *The prose of the trans-Siberian and of the Little Jehanne of France*

> (. . .) In Siberia thundered the cannon, it was war
> Hunger, cold, plague, cholera
> And the silty waters of Love swept along millions of the decaying dead.

Cendrars in the twentieth century, the apocalyptic prophesy of Saint John

> Rev. 6.8. And power was given unto them over the fourth part of the earth, to kill with sword, and with hunger, and with death, and with the beasts of the earth.

And the circle is closed. In *Heart of Darkness*, Joseph Conrad has his fearsome and dark hero, Kurtz, say this last word, as he is dying of a fever: "The horror! the horror!" [21].

But where is the garden of Eden?

Where are the snows of yesteryear? [97]

Artists have often showed us the horror, but have more often offered hope.

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INDEX

Encyclopedia of Infectious Diseases: Modern Methodologies, by M. Tibayrenc

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MICHEL TIBAYRENC, MD, PhD, is Director of Research in the Genetics of Infectious Diseases Unit of the Institut de Recherche pour le Développement in France. Currently, he is the IRD representative in Thailand. The principal organizer of the international congresses for the Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases (MEEGID) Society, Dr. Tibayrenc is also the founder and Editor-in-Chief of the journal *Infection, Genetics and Evolution*.

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