EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.7, No.1, Jan–Feb 2001

Mad Cow Disease

Pesticides and Public Health

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Search EMERGING INFECTIOUS DISEASES at www.cdc.gov/eid

In Index Medicus/Medline, Current Contents, Excerpta Medica, and other databases

Editorial Board

Dennis Alexander, Addlestone Surrey, United Kingdom (2003)
Ban Allos, Nashville, Tennesee, USA (2003)
Michael Apicella, Iowa City, Iowa, USA (2003)
Abdu F. Azad, Baltimore, Maryland, USA (2002)
Johan Bakken, Duluth, Minnesota, USA (2001)
Ben Beard, Atlanta, Georgia, USA (2003)
Barry J. Beaty, Ft. Collins, Colorado, USA (2002)
Guthrie Birkhead, Albany, New York, USA (2001)
Martin J. Blaser, New York, New York, USA (2002)
S.P. Borriello, London, United Kingdom (2002)
Donald S. Burke, Baltimore, Maryland, USA (2001)
Charles Calisher, Ft. Collins, Colorado, USA (2001)
Arturo Casadevall, Bronx, New York, USA (2002)
Thomas Cleary, Houston, Texas, USA (2001)
Anne DeGroot, Providence, Rhode Island, USA (2003)
Vincent Deubel, Lyon, France (2003)
J. Stephen Dumler, Baltimore, Maryland, USA (2002)
Durland Fish, New Haven, Connecticut, USA (2002)
Richard L. Guerrant, Charlottesville, Virginia, USA (2002)
Scott Halstead, Arlington, Virginia, USA (2001)
Seyed Hasnain, Hyderabad, India (2002)
David L. Heymann, Geneva, Switzerland (2001)
Dagmar Hulìnskà, Prague, Czech Republic (2001)
Sakae Inouye, Tokyo, Japan (2003)
Peter B. Jahrling, Frederick, Maryland, USA (2002)
Mohamed A. Karmali, Guelph, Ontario, Canada (2002)
Charles King, Cleveland, Ohio, USA (2003)
Bruce R. Levin, Atlanta, Georgia, USA (2002)
Myron Levine, Baltimore, Maryland, USA (2001)
Stuart Levy, Boston, Massachusetts, USA (2002)
Thomas J. Marrie, Edmonton, Alberta, Canada (2003)
John E. McGowan, Jr., Atlanta, Georgia, USA (2002)
Patrick S. Moore, New York, New York, USA (2002)
Philip P. Mortimer, London, United Kingdom (2002)
Fred A. Murphy, Davis, California, USA (2001)
Barbara E. Murray, Houston, Texas, USA (2002)
P. Keith Murray, Ames, Iowa, USA (2003)
James M. Musser, Hamilton, Missouri, USA (2002)
Rosanna W. Peeling, Geneva, Switzerland (2001)
David H. Persing, Seattle, Washington, USA (2002)
Richard Platt, Boston, Massachusetts, USA (2001)
Didier Raoult, Marseille, France (2002)
Leslie Real, Atlanta, Georgia, USA (2003)
David Relman, Palo Alto, California, USA (2002)
Rebecca Rico-Hesse, San Antonio, Texas, USA (2001)
Pierre Rollin, Atlanta, Georgia, USA (2003)
Nancy Rosenstein, Atlanta, Georgia, USA (2003)
Connie Schmaljohn, Frederick, Maryland, USA (2001)
Robert Shope, Galveston, Texas, USA (2002)
Bonnie Smoak, Bethesda, Maryland, USA (2001)
Rosemary Soave, New York, New York, USA (2001)
P. Frederick Sparling, Chapel Hill, North Carolina, USA (2001)
G. Thomas Strickland, Baltimore, Maryland, USA (2001)
Jan Svoboda, Prague, Czech Republic (2001)
Robert Swanepoel, Sandringham, South Africa (2002)
Phillip Tarr, Seattle, Washington, USA (2001)
Lucy Tompkins, Stanford, California, USA (2001)
Timothy Tucker, Cape Town, South Africa (2003)
Elaine Tuomanen, Memphis, Tennessee, USA (2002)
David Walker, Galveston, Texas, USA (2002)
Mary E. Wilson, Cambridge, Massachusetts, USA (2001)

Editors

Joseph E. McDade, Editor-in-Chief
Atlanta, Georgia, USA

Stephen S. Morse, Perspectives Editor
New York, New York, USA

Brian W.J. Mahy, Perspectives Editor
Atlanta, Georgia, USA

Phillip J. Baker, Synopses Editor
Bethesda, Maryland, USA

Stephen Ostroff, Dispatches Editor
Atlanta, Georgia, USA

Patricia M. Quinlisk, Letters Editor
Des Moines, Iowa, USA

Polyxeni Potter, Managing Editor
Atlanta, Georgia, USA

International Editors

Patrice Courvalin
Paris, France

Keith Klugman
Johannesburg, Republic of South Africa

Takeshi Kurata
Tokyo, Japan

S.K. Lam
Kuala Lumpur, Malaysia

John S. MacKenzie
Brisbane, Australia

Hooman Momen
Rio de Janeiro, Brazil

Sergey V. Netesov
Novosibirsk Region, Russian Federation

V. Ramalingaswami
New Delhi, India

Diana Walford
London, United Kingdom

Production Editors

Maria T. Brito
Kevin Burlison
Teresa M. Hood
Anne D. Mather
Ava W. Navin

Electronic Access

Retrieve the journal electronically on the World Wide Web (WWW) at http://www.cdc.gov/eid or from the CDC home page (http://www.cdc.gov).

Announcements of new table of contents can be automatically e-mailed to you. To subscribe, send an e-mail to listserv@cdc.gov with the following in the body of your message: subscribe EID-TOC.

Emerging Infectious Diseases

Emerging Infectious Diseases is published six times a year by the National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC), 1600 Clifton Road, Mailstop D 61, Atlanta, GA 30333, USA. Telephone 404-371-5329, fax 404-371-5449, e-mail eideditor@cdc.gov.

All material published in Emerging Infectious Diseases is in the public domain and may be used and reprinted without special permission; proper citation, however, is appreciated.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

∞ Emerging Infectious Diseases is printed on acid-free paper that meets the requirements of ANSI/NISO 239.48-1992 (Permanence of Paper).

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.7, No.1, Jan–Feb 2001

The journal is distributed electronically and in hard copy and is available **at no charge**.

☐ YES, I would like to receive Emerging Infectious Diseases.

Please print your name and business address in the box and return by fax to 404-371-5449 or mail to
EID Editor
CDC/NCID/MS D 61
1600 Clifton Road, NE
Atlanta, GA 30333

Moving? Please give us your new address (in the box) and print the number of your old mailing label here__________

EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.7, No.1, Jan–Feb 2001

Cover: Detail of The Bull (1647) by Paulus Potter (1625-1654). Reproduced with permission of the Mauritshuis, The Hague, the Netherlands. See p. 168.

International Update

International Editor's Update—Russia .... 1 S.V. Netesov & J.L. Conrad

Perspectives

Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease: Background, Evolution and Current Concerns .... 6 P. Brown et al.

Pesticides and Public Health: Integrated Methods of Mosquito Management .... 17 R.I. Rose

Synopses

Quinolone and Macrolide Resistance in *Campylobacter jejuni* and *C. coli*: Resistance Mechanisms and Trends in Human Isolates .... 24 J. Engberg et al.

Geographic Subdivision of the Range of the Malaria Parasite *Plasmodium vivax* .... 35 J. Li et al.

Research

Transferable Plasmid-Mediated Resistance to Streptomycin in a Clinical Isolate of *Yersinia pestis* .... 43 A. Guiyoule et al.

Nested Polymerase Chain Reaction for Amplification of the *Cryptosporidium* Oocyst Wall Protein Gene .... 49 S. Pedraza-Díaz et al.

Preoperative Drug Dispensing as Predictor of Surgical Site Infection .... 57 K.S. Kaye et al.

Lack of Evidence of Endogenous Avian Leukosis Virus and Endogenous Avian Retrovirus Transmission to Measles, Mumps, and Rubella Vaccine Recipients ..... 66 A.I. Hussain et al.

A Flea-Associated Rickettsia Pathogenic for Humans .... 73 D. Raoult et al.

Gastroenteritis in Sentinel General Practices, the Netherlands .... 82 M.A.S. de Wit et al.

Active Bacterial Core Surveillance of the Emerging Infections Program Network .... 92 A. Schuchat et al.

Letters

High-Level Ciprofloxacin Resistance in *Neisseria gonorrhoeae*: First Report from Israel 158
M. Dan et al.

An Unusual Bacterium Causing a Brain Abscess 159
D.N. Atapattu et al.

First Glycopeptide-Resistant *Enterococcus faecium* Isolate, from Blood Culture in Ankara, Turkey 160
A. Basustaoglu et al.

Antimicrobial-Drug Use and Methicillin-Resistant *Staphylococcus aureus* 161
D.L. Monnet & N. Frimodt-Møller

Lack of Evidence for Chloramphenicol Resistance in *Neisseria meningitidis*, Africa 163
M.L.C. Tondella et al.

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.7, No.1, Jan–Feb 2001

Letters, cont'd.

Iron Loading and Disease Surveillance 164
M. Reyes & G. Imperatore

Reply to Dr. Reyes 165
E.D. Weinberg

Commentary

Adventitious Viral Genomes in Vaccines but Not in Vaccinees 153
R.A. Weiss

Research Update

Strengthening National Preparedness for Smallpox: An Update 155
J.W. LeDuc & P.B. Jahrling

The Cover

Detail of The Bull by Paulus Potter 168

Research, cont'd.

Emerging Chagas Disease: Trophic Network and Cycle of Transmission of *Trypanosoma cruzi* from Palm Trees in the Amazon .... 100 — A.R.L. Teixeira et al.

Persistence and Variability of *Stenotrophomonas maltophilia* in Cystic Fibrosis Patients, Madrid, 1991-1998 .... 113 — S. Valdezate et al.

Hospital Control and Multidrug-Resistant Pulmonary Tuberculosis in Female Patients, Lima, Peru .... 123 — F.F. Willingham et al.

Dispatches

Outbreak of West Nile Infection, Volgograd Region, Russia, 1999 .... 128 — A.E. Platonov et al.

Rapid Identification of *Corynebacterium diphtheriae* Clonal Group Associated with Diphtheria Epidemic, Russian Federation .. 133 — S. Kombarova et al.

Shigella spp. Surveillance in Indonesia: The Emergence or Reemergence of *S. dysenteriae* .... 137 — D. Subekti et al.

Tracking *Cryptosporidium parvum* by Sequence Analysis of Small Double-Stranded RNA .... 141 — L. Xiao et al.

Pathologic Studies of Fatal Cases in Outbreak of Hand, Foot, and Mouth Disease, Taiwan .... 146 — W.-J. Shieh et al.

Disseminated *Neocosmospora vasinfecta* Infection in a Patient with Acute Nonlymphocytic Leukemia .... 149 — O. Cornely et al.

News and Notes

Summary of the 5th International Conference on Legionella, Sept. 26-29, 2000 166

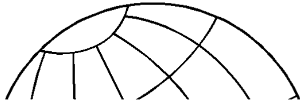
Announcement of the 4th Annual Conference on Vaccine Research, April 23-25, 2001 166

Erratum, Vol. 6 No. 4 167

Electronic Access

Retrieve the journal electronically on the World Wide Web (WWW) at http://www.cdc.gov/eid or from the CDC home page (http://www.cdc.gov).

Announcements of new table of contents can be automatically e-mailed to you. To subscribe, send an e-mail to listserv@cdc.gov with the following in the body of your message: subscribe EID-TOC.



International Editors

update

Emerging Infectious Diseases in Russia, 1990-1999

Sergey V. Netesov* and J. Lyle Conrad†
*State Research Center of Virology and Biotechnology Vector, Koltsovo, Novosibirsk Region, Russian Federation; †Medical Epidemiologist, Atlanta, Georgia, USA

Dr. Netesov, an international editor of this journal, is deputy director for research and head of the Laboratory of Molecular Biology of RNA viruses of the State Research Center of Virology and Biotechnology Vector, Koltsovo, Novosibirsk Region, Russia. His research interests include the molecular evolution and pathology of human RNA viruses, vaccine development, and the molecular epidemiology of viral hepatitis.

Russia, the world's largest country, has a population of approximately 145 million and an area of 17,075 km^2, encompassing 7 geographic, 10 time, and 3 climatic zones (1). This diversity, along with socioeconomic changes in the 1990s, substantially influences the country's infectious disease rates. We discuss infectious disease data collected since 1990 because data for earlier years are not available from officially published sources.

The system of health and epidemic surveillance in Russia, which was organized in the 1920s, has been successful in eradicating some infectious diseases and decreasing the rate of others. When epidemiologists graduate from medical school, they are assigned to sanitary epidemiologic surveillance stations throughout Russia, in oblast (state), county, and city offices. Surveillance, disease reporting, sanitation inspections, and outbreak investigations are their main functions. In 1993, the Central Moscow office of regional sanitary epidemiologic surveillance stations began publishing the monthly bulletin Population Health and Environment with collated data that are distributed within and outside Russia (www.fcgsen.ru). However, data collection is limited by inaccurate information from private clinics and diagnostic laboratories (especially those dealing with sexually transmitted diseases and HIV infection), which sometimes do not report all the results of their analyses and diagnoses.

Availability of medical statistics in hospitals and regional sanitary epidemiologic surveillance centers is still limited by shortage of personal computers, incompatible software, and slow communications, which affect the speed, reliability, and validity of data. In addition, diagnosis in polyclinics and hospitals, especially for gastrointestinal and respiratory infections, is usually based on clinical signs and symptoms rather than laboratory identification of the infectious agents or their markers. For example, data for rotaviral infections have been included in disease statistics since at least 1990, although no laboratory reagent kits have been purchased for testing for markers of these infections in most regions and no data were entered in regional reports. Another example is influenza: immunofluorescent diagnostics are performed selectively and only during outbreaks. When the number of positive samples reaches a certain level, an influenza epidemic is declared. Influenza is the diagnosis recorded in the medical charts of all patients with similar symptoms, and statistics are coded accordingly.

Russia does not yet participate in the European network for gastrointestinal diseases, the Enternet (2), although international cooperation in surveillance for such diseases as legionellosis, meningococcal infections, and malaria is improving. Increased surveillance and improved diagnostic kits could increase the reported incidence of certain diseases.

Selected Bacterial Diseases

Diphtheria

In the former Soviet Union, diphtheria was controlled through vaccination. The large

Address for correspondence: J. Lyle Conrad, 1069 Burton Dr. NE, Atlanta, GA 30329; fax: 770-922-1971; e-mail: jlconrad@mindspring.com.

increase in cases in the early 1990s was mainly due to low vaccination coverage because of a new policy, under which vaccination was not recommended for large segments of the population (3). This policy, which was introduced in the beginning of the 1990s, was rescinded in the mid-1990s. In addition, a widespread advertising campaign on television and other mass media about the adverse effects of vaccination caused widespread reluctance of the public to be vaccinated (3). As a result, by 1994, the disease rate had increased almost 30-fold. Since then, a mass vaccination campaign has been implemented in the most heavily affected regions, and by 1999 diphtheria rates had returned to the levels recorded in the early 1990s. Ministry of Health statistics (Table; 6,8) demonstrate a sharp decrease in diphtheria, which was achieved through an organized, voluntary immunization campaign on a scale unprecedented in post-Soviet Russia.

Tuberculosis (TB)

TB rates in Russia increased by 70% from 1990 to 1995, according to sanitary epidemiologic surveillance records (Table; 6,8). The disease rate in 1999 was 4.5% higher than in 1998 (4,6) (61.4/100,000 or 90,000 newly identified cases, 4,681 [5%] in children <14 years of age [6,7]). More than 25,000 persons die of TB each year (8). The highest rates are reported from Tuva, Buryatia, Khakassia, and the Tyumen, Jewish Autonomic, Perm, and Novosibirisk regions, with case rates of 266.4, 212.4, 146.8, 142.3, 137.6, 131.9, and 131 per 100,000, respectively (9). These data from the Ministry of Health likely do not reflect the disease rate among prisoners, who numbered approximately 974,000 in September 2000 (www.prison.org). The TB death rate, however, has remained stable or declined, with 16.7 per 100,000 in 1997 and 15.4 in 1998 (10).

According to the World Health Organization's definition (4), a case of TB is recorded if mycobacteria are identified directly by Ziehl-Neelsen microscopy. According to this method, the number of TB patients in Russia is approximately 20,000 (4). However, if mycobacteria are identified through culture, polymerase chain reaction, or other diagnostic methods, the estimated cumulative number of TB cases is 300,000. Additional categories of patients needing follow-up include 1 million recovered patients, 200,000 contacts of persons with newly identified cases, and 700,000 persons with positive tuberculin skin tests (4). Deterioration of living conditions in the past 10 years, including food

Table. Selected reportable diseases, Russian Federation, 1990-1999 and January-June 2000 (6,8)

Disease	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
Diphtheria*	0.98	1.53	2.65	10.25	26.9	24.1	9.2	2.76	0.98	0.6	0.23
Tuberculosis[a]	51,000	45,000	43,000	54,000	66,000	72,000	78,000	81,000	86,000	90,000	48,000
TB-all*[b]	34.2	34	35.8	42.9	48	57.9	67.5	74	76	na	na
TB-MoH data[c]	34.3	30.5	29.4	36.5	45.1	48.7	52.8	55.1	58.5	61.4	32.4
TB death rate	7.9	8.1	9.3	12.6	14.6	15.4	17	16.7	15.4	na	na
Syphilis*	5.4	7.3	12.6	32.3	82.3	172.1	254.2	266.8	226.1	179.3	81.3
Shigella*	130.4	146.6	127.3	102.4	149.9	184.2	82	57.1	78	148.4	40.1
Hepatitis A*	204.3	165.5	117.9	109.3	111.2	122.6	86.9	50.1	33.8	30.6	15.9
Hepatitis B*	21.9	17.9	18.2	22.2	27	35.2	35.8	36.5	35.8	43.3	21.7
Hepatitis C*	na	na	na	na	3.2	6.8	8.4	9.1	11.6	19.3	10.9
Measles*	12.4	13.8	12.6	50.1	20	5.2	5.4	2	4.7	5.0	2.8
Mumps*	39.2	24.6	23.6	30.1	28.1	36.1	47	69.2	97.8	48.2	19.9
Rubella*	192.6	141	na	127	245.7	186.2	115.5	121.1	304	407	247.3
Tick-borne encephalitis*	3.7	3.5	4.3	5.3	4	4	6.5	4.4	5	6.7	0.9
Influenza*	3,719	4,823	6,097	3,721	2,339	3,870	2,450	5,060	2,516	4,059	5,117
HIV, new cases	95	66	72	99	146	169	1,433	3,853	3,709	10,900	na
HIV rates*	0.06	0.04	0.05	0.07	0.1	0.11	0.97	2.6	2.5	15.9	11.4
Omsk HF[d]	29	41	7	19	11	5	2	na	7	na	na

*Rates/100,000 population.
[a]New cases (6).
[b]TB-all includes data from civilian hospitals, army clinics, prison hospitals, and medical units of other ministries (8).
[c]TB-MOH data are from civilian hospitals controlled by the Ministry of Health.
[d]Novosibirsk region only
HF = hemorrhagic fever; na = not available; MoH = Ministry of Health

shortages, poverty, and severe overcrowding in prisons, is associated with increasing TB rates. Another important factor is the spread of mycobacterial strains resistant to antibiotics, especially strains resistant to multiple drugs. Uncontrolled administration of antibiotics (e.g., in prisons) promotes emergence of resistant strains. Russia has a high rate of strains resistant to a single drug (5,10), which may lead to an increase in the number of strains resistant to multiple drugs.

Sexually Transmitted Diseases

One- to twofold annual increases in syphilis incidence were recorded by the early 1990s, with a 50-fold increase in 1997 compared with 1990 (Table); however, the rate of increase has slowed since 1996 and even decreased from 1997 to 1999 (7). These data may underestimate the incidence, as patients treated in private clinics are not fully reported in official statistics. The decrease in gonorrhea incidence, which began in 1995 and continued until 1998, when the rate of gonorrhea became half that of syphilis, may be also attributed to underreporting of these data by private clinics, which received official permission from the Ministry of Public Health to treat gonorrhea. In 1997, the regions with the highest rates of syphilis were Tuva, Khakassia, and Sakhalin, with 1,381, 1,314, and 1,217 cases per 100,000, respectively (7).

Brucellosis

In the 1990s, 300 to 700 cases of brucellosis occurred each year. No apparent long-term trends were observed.

Anthrax

Although many natural foci are located in Russia, the number of anthrax cases per year during the past 10 years has never exceeded 100 (e.g., 37 in 1998, 45 in 1999) (4).

Acute Bacterial Intestinal Infections

In 1998, dysentery rates were 37% higher than in 1997; 114,800 cases were reported, including 66,000 in children. Shigellosis (Table) shows no long-term trends. In 1998, 398,600 cases of acute intestinal infections of unknown etiology were reported, including 231,700 in children. The ratio of intestinal infections with identified and unidentified causes remains unchanged since 1990, indicating lack of progress in developing and adapting new diagnostic tools.

Other Infectious Diseases

In 1998, an increase was reported in cases of zoonotic diseases such as typhus, which increased by 10%; borreliosis (Lyme disease), which increased by 25%; and tularemia, for which a twofold increase was reported. No long-term trends were noted. A cholera outbreak was officially recorded in Russia in 1998 in Dagestan (8 cases, 17 carriers), and three isolated cases were reported elsewhere. Twenty cases of epidemic typhus and 33 cases of Brill-Zinsser disease were reported.

Resistance to Antibiotics

Antibiotic resistance has increased in Russia since antibiotics became available without prescription. In addition, a high concentration of TB patients in prisons, combined with a massive shortage of drugs in prison clinics, results in frequent self-treatment. This self-treatment leads to inappropriate selection of drugs and results in incomplete treatment, thus encouraging the emergence of drug-resistant strains. A detailed study of this situation by the Russian Academy of Medical Sciences and Academy of Sciences has just begun.

Selected Viral Diseases

Hepatitis

Rates of hepatitis A decreased during the 1990s, but rates of hepatitis B and hepatitis C increased steadily (Table). Reasons may be a sharp increase in intravenous drug use, lack of hygiene, and high-risk sexual behavior. Half the patients with acute hepatitis B and hepatitis C are 11 to 30 years of age (11). Mass vaccination of children against hepatitis B and support for the development of a vaccine for hepatitis C are needed to control these diseases.

Poliomyelitis

Poliomyelitis increased during the war in Chechnya (152 cases there in 1995) probably because of unavailability of vaccine in Chechnya during the conflict. Only six cases of acute paralysis were recorded in 1998, none of which were caused by a wild-strain virus, as shown by laboratory diagnostics (7). National immunization efforts against polio are continuing.

Measles, Mumps, and Rubella (MMR)

Measles rates have decreased considerably during the last 4 years through additional vaccination of teenagers and children at sites of mass outbreaks during 1992-94 (Table). Mumps, however, increased almost threefold from 1990 to 1998. The vaccine may have degraded during delivery or storage under inadequate conditions. In addition, funds were insufficient for mumps vaccination programs, and financial support was lacking in some regions for a second vaccination at age 6 years. In 1997, a second vaccination was recommended in the national vaccination schedule, with support from federal funds. Rates of rubella remain high, sometimes increasing to epidemic levels. Russia was one of the few European countries that did not include rubella in the schedule of mandatory, state-funded vaccines before 1997. The cost of support for disabled children born to unvaccinated mothers is much higher than the cost of vaccination; therefore, rubella vaccine should be added to the national vaccination schedule, ideally in the form of MMR.

Tick-borne encephalitis

Tick-borne encephalitis, a severe zoonotic disease, is occasionally fatal (Table). The agent is a flavivirus transmitted through tick bites. Cases vary from 5,000 to 10,000 per year. Some regional administrations fund local vaccination programs for children and adults at high risk. Adults can pay for the vaccination in most disease-endemic regions (Krasnoyarsk, Novosibirsk, Tomsk, Irkutsk, Omsk, and Kemerovo).

Omsk hemorrhagic fever

Omsk hemorrhagic fever is a zoonosis caused by a flavivirus; the infection is transmitted by muskrats during trapping. During the past 10 years, this disease has been reported only from Novosibirsk Region (Province) (Table). Of the seven cases reported in 1998, one was fatal and three were severe.

Hemorrhagic Fever with Renal Syndrome

Hemorrhagic fever with renal syndrome, caused by a representative of the Bunyaviridae family, has many foci in Russia. The number of cases ranged from 2,774 in 1990 to more than 20,000 in 1997. A large outbreak in 1997 was attributed to a surge in the population of rodents, the natural carriers of the agent. The disease rate returned to an average annual level of approximately 5,000 cases by 1998.

Rabies

In Russia, the case rate of rabies in humans remains constant (7 to 16 annual cases over the past 10 years). All cases seem to be associated with ignorance of postexposure prophylaxis as a protective measure. In 1998, animal rabies in Novosibirsk increased sharply in both domestic dogs and wild animals. The population was informed about the epidemic and the availability of vaccination if needed, and no human cases were reported despite an increase in the number of animal bites.

Influenza

The rate of influenza has been stable every year except for peaks in 1992 and 1997 (Table). In Russia, this disease is diagnosed mainly by clinical symptoms, often without laboratory confirmation; the data represent a background of 22 million to 23 million cases of acute respiratory infection with unknown etiology reported each year.

HIV Infection

The number of HIV-infected persons increased from 95 in 1990 to 3,709 in 1998, virtually doubling each year from 1993 to 1998 (Table). The number of HIV-infected patients reached 15,569 by September 1999 (12) and a report in the November 17, 2000, issue of Izvestia stated that the number of officially registered HIV-positive persons had increased to 69,120. These official statistics on HIV may reflect only 10% to 20% of the actual number of carriers (12). A recent study of the Irkutsk prison population identified more than 1,400 HIV-infected prisoners (pers. commun., office of public health, Irkutsk Region), although only 30 cases had previously been reported from the entire region.

Conclusions

Three groups of diseases cause most concern in Russia, as well as elsewhere: TB, viral parenteral hepatitis, and HIV infection. Public measures for their control in Russia are insufficient, mainly because of lack of funding for treatment, vaccine prophylaxis, and health education. Immunization of children against hepatitis B is indicated. The development and introduction of additional diagnostic tools for

markers of intestinal and respiratory infection and additional vaccination against mumps and rubella are needed. However, it is unlikely that existing public health funding will allow additional improvements in the near future.

References

1. Goskomstat Rossii, Rossiiskii Statisticheskii Ezegodnik, 1998 Goskomstat, Moscow, p. 34-35, 101-102.
2. International Surveillance Network for the enteric infections - Salmonella and VTEC 0157. Available from: URL: http://www2.phls.co.uk/.
3. Tatochenko V. Vaccinoprophylactics. Meditsinskaya Gazeta 1999;87:8-9.
4. Priimak A. The sorrowful date. Meditsinskaya Gazeta 1999;22:1-4.
5. Centers for Disease Control and Prevention. Primary Multidrug-Resistant Tuberculosis—Ivanovo Oblast, Russia. MMWR Morbid Mortal Wkly Rep 1999;48:30.
6. Zdorov'e naseleniya I sreda obitaniya (Population Health and Environment), Monthly Information Bulletin, Federal Center of Sanitary and Epidemic Surveillance, Ministry of Public Health of the Russian Federation, No. 1 (22), 1995; No 1 (34), 1996, No. 1(46), 1997; No.1(58), 1998; No.1(70), 1999; No.1 (82), 2000; No. 7(88), 2000.
7. Press release of a conference on the sanitary and epidemic situation in the Russian Federation in 1998. Zdorov'e naseleniya I sreda obitaniya (Population Health and Environment), Monthly Information Bulletin, Federal Center of Sanitary and Epidemic Surveillance, Ministry of Public Health of the Russian Federation. 1999;2:1-4.
8. Ministry of Public Health. The public health of the population of Russia and activities of public health service in 1998. Moscow; 1999. p. 55.
9. Zdorov'e naseleniya i sreda obitaniya (Population Health and Environment). Monthly Information Bulletin 1999;3:19-22.
10. Perepletchikov L. Tuberculosis made one step back but only one. Meditsinskaya Gazeta 1999;86:3.
11. Shustov AV, Maksyutov AZ, Kiselev NN, Mishin VP, Tolokonskaya NP, Robertson BJ, et al. Prevalence of hepatitis C virus infection markers and genotypes among patients of the first municipal infectious disease clinical hospital of Novosibirsk. Voprosi virusologii 2000;6:22-7.
12. Krasnoyarskii V. HIV is increasing and Moscow is not the exception. Meditsinskaya Gazeta 1999;68:10.

Bovine Spongiform Encephalopathy and Variant Creutzfeldt-Jakob Disease: Background, Evolution, and Current Concerns

Paul Brown,* Robert G. Will,† Raymond Bradley,‡ David M. Asher,§ and Linda Detwiler¶

*National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA; †National Creutzfeldt-Jakob Disease Surveillance Unit, Western General Hospital, Edinburgh, Scotland; ‡Central Veterinary Laboratory, New Haw, Addlestone, UK; §Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, Maryland, USA; ¶Animal and Plant Health Inspection Service, U.S. Department of Agriculture, Robbinsville, New Jersey, USA

The epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom, which began in 1986 and has affected nearly 200,000 cattle, is waning to a conclusion, but leaves in its wake an outbreak of human Creutzfeldt-Jakob disease, most probably resulting from the consumption of beef products contaminated by central nervous system tissue. Although averaging only 10-15 cases a year since its first appearance in 1994, its future magnitude and geographic distribution (in countries that have imported infected British cattle or cattle products, or have endogenous BSE) cannot yet be predicted. The possibility that large numbers of apparently healthy persons might be incubating the disease raises concerns about iatrogenic transmissions through instrumentation (surgery and medical diagnostic procedures) and blood and organ donations. Government agencies in many countries continue to implement new measures to minimize this risk.

Bovine Spongiform Encephalopathy

> "The hungry Sheep look up, and are not fed,
> But swoln with wind, and the rank mist they draw
> Rot inwardly, and foul contagion spread…"
> John Milton, *Lycidas* (1637)

Bovine spongiform encephalopathy (BSE) or "mad cow disease" appears to have originated from scrapie, an endemic spongiform encephalopathy of sheep and goats that has been recognized in Europe since the mid-18th century (1). It has since spread to most sheep-breeding countries and is widespread in the United Kingdom (UK), where until 1988 the rendered carcasses of livestock (including sheep) were fed to ruminants and other animals as a protein-rich nutritional supplement.

Address for correspondence: Paul Brown, Building 36, Room 4A-05, National Institutes of Health, 36 Convent Drive, MSC 4122 Bethesda, MD 20892-4122; fax: 301-496-8275; e-mail: brownp@ninds.nih.gov.

During rendering, carcasses from which all consumable parts had been removed were milled and then decomposed in large vats by boiling at atmospheric or higher pressures, producing an aqueous slurry of protein under a layer of fat (tallow). After the fat was removed, the slurry was desiccated into a meat and bone meal product that was packaged by the animal food industry and distributed to owners of livestock and other captive animals (e.g., zoo and laboratory animals, breeding species, pets).

Although elements of the ensuing story are still disputed (including its origin from scrapie, rather than from unrecognized endemic BSE), it appears likely that changes in the rendering process that had taken place around 1980 allowed the etiologic agent in infected carcasses to survive, contaminate the protein supplement, and infect

cattle. Cattle carcasses and carcass wastes were then recycled through the rendering plants, increasing the levels of the now cattle-adapted pathogen in the protein supplement and eventually causing a full-scale BSE epidemic (2-5).

Recognition of this source of infection has led to a series of countermeasures taken by the UK and other countries to break the cycle of cattle reinfection, restrict the geographic spread of disease, and eliminate potential sources of new infections (Figure, Appendix). Probably the single most important measure in the UK was the imposition in 1988 of a ruminant protein feed ban that by 1992 began to bring the epidemic under control. However, the loss of nearly 200,000 diseased cattle, followed by preemptive slaughter and destruction of nearly four and a half million asymptomatic cattle >30 months of age, has crippled the British livestock industry and also affected the tallow, gelatin, and pharmaceutical industries, all of which make bovine-derived products.

BSE is not restricted to the UK. Cases have occurred in many other countries as a result of imported live animals or livestock food supplements (Table 1). In some countries, including the UK, the incidence of new cases is decreasing, but in other countries–France, Portugal, Germany, Spain, and the Republic of Ireland–the incidence appears to be increasing, or initial cases have only recently appeared. The explanation for this phenomenon is most probably improved case ascertainment (supported by active surveillance and immunologic methods), but new infections from contaminated feed intended for other species (e.g., pigs and poultry) may also be a contributing factor. Although in many countries, BSE has been identified in native-born cattle, no indigenous index case has been reported outside the UK (i.e., no case originating de novo or from cow-to-cow transmission). Whatever the origin of these cases, recycling of their contaminated tissues through livestock feed supplements could have occurred in the same way as in the UK.

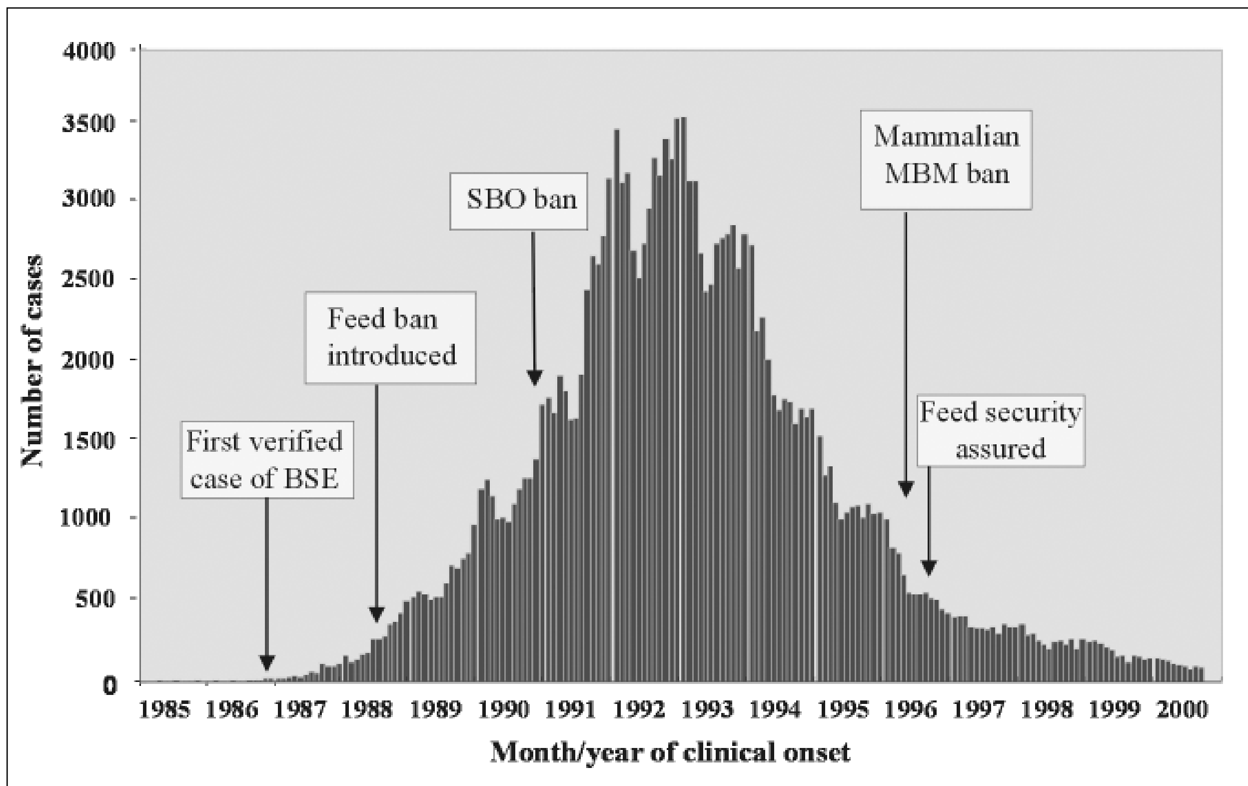


Figure. Time course of epidemic bovine spongiform encephalopathy in the United Kingdom, 1986-2000, with dates of major precautionary interventions. The mammalian ban on meat and bone meal in March 1996 extended a 1994 ban for farmed food animal species to include all mammalian species.
SBO = specified bovine offals (brain, spinal cord, thymus, tonsil, spleen, and intestines from cattle >6 months of age); MBM = meat and bone meal (protein residue produced by rendering).

Table 1. Reported cases of bovine spongiform encephalopathy in the United Kingdom and other countries (as of December 2000)

Country	Native cases	Imported cases	Total cases
United Kingdom	180,376[a]	-	180,376
Republic of Ireland	487	12	499
Portugal	446	6	452
Switzerland[b]	363	-	363
France[b]	150	1	151
Belgium	18	-	18
Netherlands	6	-	6
Liechtenstein	2	-	2
Denmark	1	1	2
Luxembourg	1	-	1
Germany	3	6	9
Oman	-	2	2
Italy	-	2	2
Spain[c]	-	2	2
Canada	-	1	1
Falklands (UK)	-	1	1
Azores (Portugal)[d]	-	1	1

Data from Organization of International Epizootics (Paris) and Ministry of Agriculture, Fisheries, and Food (UK).
[a]Includes 1,287 cases in offshore British islands
[b]Includes cases detected by active surveillance with immunologic methods
[c]Origin and dates of imported cases are under investigation.
[d]Case imported from Germany.

BSE has not occurred in the United States or other countries that have historically imported little or no live cattle, beef products, or livestock nutritional supplements from the UK. Even though rendering procedures in other countries underwent changes similar to those in the UK during the late 1970s, BSE has apparently emerged solely within the UK. The most plausible explanation is that the proportion of sheep in the mix of rendered animal carcasses and the proportion of scrapie infections in such sheep were probably higher in the UK than elsewhere. These proportions were apparently sufficient to bring very low levels of the etiologic agent in batches of rendered carcasses over the threshold of transmission in the UK but not in other countries (5). An alternative explanation proposed in the recent Report of the BSE Inquiry (6) is that a pathogenic mutation occurred in cattle in the 1970s.

Either of these two hypotheses satisfies the need for an etiologic "seed" to survive the altered rendering process and escalate through recycling of an ever-larger number of infected carcasses. However, the bovine origin hypothesis assumes that a mutation occurred only in the UK and not in other countries where similar rendering processes would also have led to epidemic BSE if mutations were occurring. In humans, mutations have occurred all over the world, not just in the UK, and there is no reason to suppose that humans differ in this respect from other mammalian species. It would therefore be peculiar if the UK had the misfortune to host the cattle world's only mutation.

Variant Creutzfeldt-Jakob Disease (vCJD)

> How soon hath Time, the subtle thief of youth,
> Stol'n on his wing my three and twentieth year!
> John Milton, *Sonnet* (1632)

Within weeks of identification of the first case of BSE, concern was expressed about human risk (7-13), and as the epidemic unfolded, a series of measures was taken to eradicate BSE and prevent potentially infected tissues from reaching the human food chain (Appendix). A surveillance unit to monitor CJD was established in the UK in May 1990, and 3 years later, surveillance was extended to several other European countries, coordinated through the European Union. By this means it was hoped that any change in the epidemiology of CJD in the UK could be detected quickly and that the significance of the change could be assessed by comparison with the epidemiology of CJD in continental Europe.

Concern was heightened by the discovery that some exotic zoo ungulates, as well as domestic and captive wild cats, were becoming infected (14-18). The ungulates and domestic cats had also been fed diets supplemented by meat and bone meal, and the wild cats had been fed uncooked tissues, including cattle heads and spines. The possibility could therefore not be ignored that the disease might also cross the species barrier to humans from the consumption of beef or dairy products, or perhaps from occupational contact with cattle by ranchers, dairymen, or slaughterhouse workers.

What muted concerns about human infection was the presumption that BSE originated from scrapie, and scrapie was not a human pathogen. Nevertheless, even those who considered human risk to be remote acknowledged that scrapie might unpredictably show an altered host range

after passage through cattle. Experimental precedents for such behavior were well known: passage of mouse-adapted strains of scrapie through hamsters altered their transmissibility on back passage to rodents (19,20); human strains of kuru or CJD did not transmit to ferrets or goats until passaged through primates or cats (21); and a bovine strain of BSE did not transmit to hamsters until passaged through mice (22). Alternatively, if BSE originated from a spontaneous mutation in cattle, experimental studies of species susceptibility to this new strain of transmissible spongiform encephalopathy (TSE) had not sufficiently advanced to predict that humans would not be susceptible. Nevertheless, during the 10 years after the first case of BSE was identified, cases of CJD did not increase in groups at high risk and continued to occur in the general population with the same spectrum of clinical and neuropathologic features as before the appearance of BSE.

Then, from May to October 1995, the CJD Surveillance Unit was notified of three cases of CJD in patients 16, 19, and 29 years of age (23,24). On neuropathologic examination, all three patients had amyloid plaques, which was unexpected in view of their occurrence in only 5%-10% of sporadic cases of CJD. The comparative youth of the patients and this unusual neuropathologic finding prompted a search for similar features in patients whose deaths might have been attributed to other diagnoses. In particular, cases of subacute sclerosing panencephalitis (SSPE) were scrutinized in view of a report from Poland that cases of CJD in three young patients had been identified by SSPE surveillance (25). No such cases were found in a review of the UK SSPE register.

If CJD in young patients was not being obscured by misdiagnosis, perhaps it reflected increased physician awareness through publicity surrounding BSE and iatrogenic CJD in recipients of contaminated growth hormone, or the active CJD surveillance program instituted in the UK, or the availability of genetic and proteinase-resistant protein (PrP) immunocytochemistry. Although all these factors may have contributed to ascertainment bias, most of the excess cases were in older age groups, in which CJD was now being diagnosed more often than in earlier decades.

By December 1995, the Surveillance Unit had been informed of 10 suspected cases of CJD in persons <50 years of age. Some were found to have sporadic or familial CJD or some other disease; however, two of the patients, ages 29 and 30 years, were later confirmed neuropathologically to have CJD and, like the previous three CJD patients, had extensive plaque deposition. As of January 1, 1996, the relationship between these cases and BSE began to excite suspicion but remained tentative because critical information judged necessary to establish a probable connection was still missing (Table 2).

During January, two additional cases of CJD in young patients were neuropathologically confirmed, and a distinctive clinical syndrome associated with plaque formation was beginning to emerge: young age at onset, early psychiatric symptoms, prominent ataxia, absence of periodic electroencephalographic activity, and a comparatively prolonged illness. However, each of these features, alone or in combination, may also be seen in classic sporadic or familial CJD. Caution was further justified by a review of the records of pre-1980 CJD patients in the UK, which identified three young patients who shared some of these features, and by the results of an inquiry about young patients with CJD in other European countries, which showed an age distribution similar to that in the UK. A major concern was that these seven apparently similar cases might represent a heterogeneous group of patients with sporadic and familial forms of CJD.

Table 2. Evolving assessment of criteria used to link bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease.

	Assessment through early 1996				
Criteria	Jan 1	Feb 1	Mar 1	Mar 8	Mar 20
Novel clinical phenotype	Uncertain	Possible	**Probable**	Probable	Probable
Novel neuropathologic phenotype	Uncertain	Possible	**Probable**	Probable	Probable
Distinct from pre-1980 cases in UK	Unknown	Possible	**Probable**	Probable	Probable
No association with PRNP mutations	Uncertain	Uncertain	Uncertain	**Probable**	Probable
Distinct from cases outside UK	Unknown	Unknown	Unknown	Possible	**Probable**

Full comparative neuropathologic examination of both pre- and post-1980 cases of CJD in young persons was needed, along with *PRNP* gene sequence analysis of as many cases as possible.

During February 1996, an additional case was referred to the Surveillance Unit with a clinical evolution similar to that of the previous seven patients, and neuropathologic examination of recent and historical cases confirmed that the recent cases were indeed distinctive. In particular, a morphologically unusual form of plaque was present in all cases: the florid or "daisy" plaque in which an amyloid core was surrounded by "petals" of spongiform change. As of March 1, despite the likelihood that this group of patients had a "new variant" of CJD, it was still unclear whether mutations were involved and whether such a syndrome was also occurring outside the UK–both points essential to confirming the association of this variant disease with exposure to BSE.

On March 4, genetic analysis was completed for six of the cases, and no pathogenic mutation was identified. These results effectively ruled out a genetic cause for the syndrome (although they did not rule out a genetic predisposition) and left the only remaining uncertainty–the geographic distribution of the variant phenotype–to be resolved by the European CJD surveillance system. The answer came by March 20: none of the young CJD patients in other European countries had the clinical and neuropathologic features of the UK cases. In the preceding week, two more variant cases had been neuropathologically confirmed, and a report on the entire group of 10 cases concluded that an unrecognized variant of CJD occurring only in persons <45 years of age was probably due to exposure to BSE (26).

This link has now been convincingly established in laboratory studies showing identical, distinctive biological and molecular biological features of the pathologic agent isolated from BSE-infected cattle and human cases of vCJD (27-29). The source of contamination appears to have been beef. However, muscle has never been reproducibly shown to contain the infectious agent in any form of spongiform encephalopathy, whatever the affected species, and thus, infection most probably resulted from beef products contaminated by nervous system tissue. Contamination could have occurred in any of the following ways: cerebral vascular emboli from cranial stunning instruments used to immobilize cattle before killing by exsanguination; contact of muscle with brain or spinal cord tissue by saws or other tools used during slaughter; inclusion of paraspinal ganglia in cuts of meat containing vertebral tissue (e.g., T-bone steaks); and perhaps most importantly, the presence of residual spinal cord and paraspinal ganglia tissue in the paste of "mechanically recovered meat" (a carcass compression extract) that could legally be added to cooked meat products such as meat pies, beef sausages, and various canned meat preparations. Measures have since been taken to eliminate these sources of potential contamination and limit the consequences of any contamination that may already have occurred (Appendix).

Although the amount of infectious tissue ingested must be a critical determinant for the transmission of BSE to humans in the form of vCJD, the human genotype at polymorphic codon 129 of the PRNP gene appears to play an important role in susceptibility to infection. The encoding alternatives, methionine (Met) and valine (Val), are distributed in the general Caucasian population in the approximate proportions of 50% Met/Val, 40% Met/Met, and 10% Val/Val. All 76 vCJD patients tested have been homozygous for methionine, and the apparently single infecting strain of BSE may not be able to replicate in any other human genotype. However, it is also possible that (as in the analogous oral infection of kuru and in peripheral iatrogenic CJD infections) heterozygotes are comparatively resistant to disease and become ill after longer incubation periods than those of homozygotes (30-33).

Predictions about the vCJD Outbreak

> Think not but that I know these things; or think
> I know them not: not therefore am I short
> Of knowing what I ought.
>
> John Milton, *Paradise Regained* (1671)

The onset of illness in the first case of vCJD occurred in early 1994, nearly a decade after the first case of BSE was recognized in cattle. Assuming that the earliest appearance of vCJD reflects the earliest exposure to BSE, this incubation period is consistent with those following peripheral infections in experimental animals and in cases of iatrogenic CJD in

humans. Through the end of November 2000, the overall tally was 87 definite or probable cases of vCJD in the UK, 2 confirmed and 1 probable case in France, and a single confirmed case in the Republic of Ireland (Table 3). The Irish patient had lived for some years in England; however, none of the French patients had lived in or visited the UK, so their infection must have come either from beef or beef products imported from the UK (approximately 5%-10% of the beef consumed in France) or from BSE-affected cattle in France. From a European standpoint, it would be much more troubling if imported beef were the source, as most European countries also imported beef or beef products from the UK, although in smaller quantities.

Unlike the BSE epidemic, the vCJD outbreak has shown only a modest increase during its first 6 years, and the number of cases with onsets in 2000 remains well below the previous year's total, although additional cases will certainly be identified in coming months. The difference between BSE and vCJD may be due to the fact that, in humans, recycling of infected tissue has not occurred, and thus the epidemic will evolve much more slowly than in cattle, or the difference may indicate a limited outbreak in humans due to very small infectious doses that, except in genetically susceptible persons, cannot surmount the combined effects of a species barrier and comparatively inefficient route of infection.

Much of the lingering uncertainty about the extent of the vCJD outbreak is attributable to the fact that the incubation period of vCJD is unknown. If the average incubation period is 10 to 15 years, the earliest patients with vCJD would have been infected in the early 1980s, when BSE was still silently incubating in small but increasing numbers of cattle. In this case, the large increase in human exposure to contaminated tissues during the late 1980s could lead to a parallel increase in cases of vCJD during the next few years. If, however, the average incubation period of vCJD is 5 to 10 years, the earliest human infections would have begun in the mid- to late 1980s, when exposure to BSE was maximal. In this case, the outbreak of vCJD should remain small because of measures to eliminate both animal and human exposure to BSE instituted from 1987 to 1997. Depending on assumptions about the incubation period and other variables, mathematical modeling predicts that the total extent of the outbreak could range from fewer than one hundred to hundreds of thousands of cases (34-37).

If large numbers of infected persons are silently incubating the disease, the potential for human-to-human iatrogenic spread of vCJD is very real. Such apparently healthy persons would be subject to the same kinds of medical and surgical procedures experienced by the general population, including endoscopies, vascular catheterizations, operations for trauma or illness, and blood and organ donations. If, as suspected, the amount and distribution of the infectious agent in tissues of persons with vCJD is greater than in other forms of CJD, the exposure of medical and surgical instruments to possibly infectious internal tissues and the transfer of tissues as grafts and transplants become a matter of much greater concern than the nearly negligible risk currently posed by cases of sporadic CJD.

Table 3. Chronology of variant Creutzfeld-Jakob disease (vCJD) in the United Kingdom and other European countries, as of December 2000

Year of onset	United Kingdom	France	Ireland
1994	8	1	
1995	10		
1996	11		
1997	14		
1998	17		
1999[a]	20 (+4)	1 (+1)	1
2000[a]	1 (+2)		

[a]Parentheses indicate still-living persons with probable vCJD or deceased persons whose diagnoses have not yet been confirmed by neuropathologic examination. In 2000, additional cases have been identified that do not yet meet the minimum clinical criteria for a premortem diagnosis of "probable" vCJD. Dates are for year of onset of illness, not year of death.

Recent and Future Policy Decisions

> A little onward lend thy guiding hand
> To these dark steps, a little further on…
>
> John Milton, *Samson Agonistes* (1671)

Several governments have implemented policies to minimize the risk for human-to-human disease transmission through blood donations from apparently healthy persons who may be in the incubation phase of vCJD. In the

UK, where whole blood or blood products from some persons who later died of vCJD have been administered to others, all plasma is imported and all blood from UK donors is filtered to eliminate leukocytes, which are the most likely carriers of infectivity in blood (38-40). In the United States, a blood donor policy excludes donations from anyone who has lived in or visited the UK for a cumulative period of 6 months or more during 1980 to 1996. The 6-month period was based on the fact that >80% of total US person-years in the UK would be excluded and that the 2%-3% deficit of blood donors resulting from the deferral could be absorbed by the blood banking industry without undue shortages. Several countries (Canada, Australia, New Zealand, Switzerland, Japan, and Germany) have since applied these criteria and formulated similar policies.

Because of the possibility of widespread infection in the UK, concern extends beyond blood and organ donors to the safe use of medical and surgical instruments, particularly those used in neurosurgery and ophthalmic surgery. In the absence of a screening test, a zero-risk policy is untenable because it would require termination of the national organ donor program. A compromise might be the temporary deferral of organ donors–or perhaps only corneal donors–younger than 30 or 40 years of age. However, this measure might so diminish (and panic) the donor population as to be inadvisable. Similar considerations apply to invasive medical and surgical procedures: sound medical practice cannot be suspended on the basis of a theoretical risk for vCJD, and it would be unethical to deny needed procedures to persons suspected of having CJD. Under the circumstances, disposable instruments should be used whenever possible, and a standard sterilization protocol for reusable instruments should be implemented that includes the most stringent possible disinfectants (e.g., the combined use of 1 N sodium hydroxide and autoclaving at 134°C, as recommended in the recent World Health Organization guidelines on infection control for CJD [41]). No effective sterilization procedure yet exists for instruments or instrument parts too delicate to withstand these harsh measures. Each such instrument must be disinfected to the maximum extent possible, for example by washing repeatedly with detergent/proteinase solutions and exposing the washed instruments to less harsh chemicals (e.g., 6 M urea or 4 M guanidinium thiocyanate) that have shown moderate to good disinfection of TSE tissue extracts (42-44).

An equally important issue is whether the bovine-adapted scrapie agent has recrossed the species barrier to sheep, carrying its newly acquired ability to infect humans. The only reliable method to distinguish strains of TSE is a time-consuming comparison of incubation periods and topographic features of brain lesions after injection into different strains of inbred mice (28). Glycotyping of PrP strains extracted from diseased brain tissue is much faster but has not been convincingly shown to discriminate reliably between BSE and scrapie. Moreover, neither method has been used to test a sheep-adapted strain of BSE (that is, after multiple passages through sheep), which might have lost the distinguishing characteristics found on primary passage from cow to sheep.

If BSE did back-cross to sheep fed the same contaminated meat and bone meal that infected cattle, the consequences for humans will remain limited to the same period of risk as BSE–roughly 1980 through 1996–unless sheep BSE, like sheep scrapie, can be horizontally or maternally transmitted. Without a test to discriminate between the two diseases, there would be no defense against the development of endemic BSE in sheep and the consequent risk for human infection from sheep as well as cows. Therefore, global elimination of animal TSEs must seriously be considered.

Such a goal is more practical than it was even a few years ago. National programs to eliminate scrapie have historically relied on selective slaughter of blood lines or in some cases entire flocks in which scrapie was identified, and all such attempts have failed. Molecular genetic tools are now available to guide scrapie-resistance breeding programs that until recently depended on field observation and classical genetics, and immunologic tools can detect preclinical scrapie infection in tonsils, third eyelids, and possibly blood (45-48). The environmental durability of TSE pathogens will make their eradication difficult (49,50); however, the global elimination of TSE in sheep and other animals is a goal worth the expense, effort, and patience that will be needed for its achievement.

Dr. Brown is Senior Research Scientist in the Laboratory of Central Nervous System Studies at the National Institutes of Health. His most recent research focuses on the problem of iatrogenic Creutzfeldt-Jakob disease and on the potential for disease transmission through the administration of blood or blood products. He serves as consultant to the European CJD surveillance program and as Chair of TSEAC, the transmissible spongiform encephalopathy advisory committee of the United States Food and Drug Administration.

References

1. Brown P, Bradley R. 1755 and all that: a historical primer of transmissible spongiform encephalopathy. Br Med J 1998; 317:1688-92.
2. Wells GAH, Scott AC, Johnson CT, Gunning RF, Hancock RD, Jeffrey M, et al. A novel progressive spongiform encephalopathy in cattle. Vet Rec 1987;121:419-20.
3. Collee JG, Bradley R. BSE: a decade on-Part 1. Lancet 1997; 349:636-42.
4. Collee JG, Bradley R. BSE: a decade on-Part 2. Lancet 1997; 349:715-21.
5. Brown P. The risk of bovine spongiform encephalopathy ("mad cow disease") to human health. JAMA 1997;278:1008-11.
6. The BSE inquiry: report, evidence and supporting papers of the inquiry into the emergence and identification of Bovine Spongiform Encephalopathy (BSE) and variant Creutzfeldt-Jakob Disease (vCJD) and the action taken in response to it up to 20 March 1996. Lord Phillips of Worth Matravers, Chairman. London: The Stationery Office. October 26, 2000.
7. Holt TA, Phillips J. Bovine spongiform encephalopathy. Br Med J 1988;296:1581-2.
8. BSE and scrapie: agents for change [editorial]. Lancet 1988;ii:607-8.
9. Taylor DM. Bovine spongiform encephalopathy and human health. Vet Rec 1989;125:413-5.
10. Dealer SF, Lacey RW. Transmissible spongiform encephalopathies: the threat of BSE to man. Food Microbiol 1990;7:253-79.
11. Kimberlin RH. Bovine spongiform encephalopathy: taking stock of the issues. Nature 1990;345:763-4.
12. Will RG. Is there a potential risk of transmission of BSE to the human population and how may this be assessed? In: Bradley R, Savey M, Marchant B, editors. Sub-acute spongiform encephalopathies. Dordrecht: Kluwer Academic Publishers; 1991. p. 179-86.
13. Brown P. The clinical epidemiology of Creutzfeldt-Jakob disease in the context of bovine spongiform encephalopathy. In: Bradley R, Savey M, Marchant B, editors. Subacute spongiform encephalopathies. Dordrecht: Kluwer Academic Publishers; 1991. p. 195-202.
14. Jeffrey M, Wells GAH. Spongiform encephalopathy in a Nyala (*Tragelaphus angasi*). Vet Pathol 1988;25:398-9.
15. Fleetwood AJ, Furley CW. Spongiform enecphalopathy in an eland. Vet Rec 1990;126:408-9.
16. Wyatt JM, Pearson GR, Smerdon T, Gruffydd-Jones TJ, Wells GAH. Spongiform encephalopathy in a cat. Vet Rec 1990;126:513.
17. Kirkwood JK, Wells GAH, Wilesmith JW, Cunningham AA, Jackson SI. Spongiform encephalopathy in an Arabian oryx (*Oryx leucoryx*) and a greater kudu (*Tragelaphus strepsiceros*). Vet Rec 1990;127:418-20.
18. Willoughby K, Kelly DF, Lyon DG, Wells GAH. Spongiform encephalopathy in a captive puma (*Felis concolor*). Vet Rec 1992;131:431-4.
19. Kimberlin RH, Cole S, Walker CA. Temporary and permanent modifications to a single strain of mouse scrapie on transmission to rats and hamsters. J Gen Virol 1987;68: 1875-81.
20. Kimberlin RH, Walker CA, Fraser H. The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. J Gen Virol 1989;70: 2017-25.
21. Gibbs CJ Jr, Gajdusek DC, Amyx H. Strain variation in the viruses of Creutzfeldt-Jakob disease and kuru. In: Prusiner SB, Hadlow WJ, editors. Slow transmissible diseases of the nervous system. Volume 2. New York: Academic Press; 1979. p. 87-110.
22. Foster JD, Hope J, McConnell I, Bruce M, Fraser H. Transmission of bovine spongiform encephalopathy to sheep, goats, and mice. Ann NY Acad Sci 1994;724:300-3.
23. Britton TC, Al-Sarraj S, Shaw C, Campbell T, Collinge J. Sporadic Creutzfeldt-Jakob disease in a 16-year-old in the UK. Lancet 1995;346:1155.
24. Bateman D, Hilton D, Love S, Zeidler M, Beck J, Collinge J. Sporadic Creutzfeldt-Jakob disease in a 18-year-old in the UK. Lancet 1995;346:1155-6.
25. Kulczycki J, Jedrzejowska H, Gajkowski K, Tarnowska-Dziduszko E, Lojkowska W. Creutzfeldt-Jakob disease in young people. Eur J Epidemiol 1991;5:501-4.
26. Will RG, Ironside, JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch A, et al. A new variant of Creutzfeldt-Jakob disease in the UK. Lancet 1996;347:921-5.
27. Collinge J, Sidle KC, Heads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature 1996;383:685-90.
28. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, et al. Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. Nature 1997;389:498-501.
29. Scott MR, Will R, Ironside J, Nguyen HB, Tremblay P, DeArmond SJ, et al. Compelling transgenetic evidence for transmission of bovine spongiform encephalopathy prions to humans. Proc Natl Acad Sci U S A 1999;96:15137-42.
30. Cervenáková L, Goldfarb LG, Garruto R, Lee H-E, Gajdusek DC, Brown P. Phenotype-genotype studies in kuru: implications for new variant Creutzfeldt-Jakob disease. Proc Nat Acad Sci U S A 1998;95:13239-41.
31. Lee H-S, Brown P, Cervenáková L, Garruto RM, Alpers MP, Gajdusek DC, et al. Evidence for susceptibility of the 129MM PRNP genotype in epidemic kuru. J Infect Dis 2000, in press.
32. d'Aignaux JH, Costagliola D, Maccario J, Billette de Villemeur T, Brandel J-P, Deslys JP, et al. Incubation period of Creutzfeldt-Jakob disease in human growth hormone recipients in France. Neurology 1999;53:1197-201.

33. Brown P, Preece M, Brandel J-P, Sato T, McShane L, Zerr I, et al. Iatrogenic Creutzfeldt-Jakob disease at the millennium. Neurology 2000;55:1075-81.
34. Cousens SN, Vynnycky E, Zeidler M, Will RG, Smith PG. Predicting the CJD epidemic in humans. Nature 1997;385:197-8.
35. Ghani AC, Ferguson NM, Donnelly CA Hagenaars TJ, Anderson RM. Epidemiological determinants of the pattern and magnitude of the vCJD epidemic in Great Britain. Proc R Soc London (Series B) 1998;265:2443-52.
36. Donnelly CA, Ferguson NM. Predictions and scenario analysis for vCJD. In: Statistical aspects of BSE and vCJD: models for an epidemic. Boca Raton (FL): CRC Press LLC 1999:163-94.
37. Ghani AC, Ferguson NM, Donnelly CA, Anderson RM. Predicted vCJD mortality in Great Britain. Nature 2000;406:583-4.
38. Brown P. Can Creutzfeldt-Jakob disease be transmitted by transfusion? Curr Opin Hematol 1995;76:472-7.
39. Brown P, Cervenáková L, McShane LM, Barber P, Rubenstein R, Drohan WN. Further studies of blood infectivity in an experimental model of tranmissible spongiform encephalopathy, with an explanation of why blood components do not transmit disease in humans. Transfusion 1999;39: 1169-78.
40. Brown P, Cervenáková L. Reply to a letter to the editor. Transfusion 2000;40:754-5.
41. WHO infection control guidelines for transmissible spongiform encephalopathies: report of a WHO Consultation. WHO/CDS/CSR/APH/2000.3. Geneva: March 23-26, 1999.
42. Kimberlin RH, Walker CA. Competition between strains of scrapie depends on the blocking agent being infectious. Intervirology 1985;23:74-81.
43. Manuelidis L. Decontamination of Creutzfeldt-Jakob disease and other transmissible agents. J Neurovirol 1997;3:62-5.
44. Pocchiari M, Peano S, Conz A, Eshkol A, Maillard, Brown P, et al. Combination ultrafiltration and 6 M urea treatment of human growth hormone effectively minimizes risk from potential Creutzfeldt-Jakob disease virus contamination. Horm Res 1991;35:161-6.
45. Roels S, Vanopdenbosch E, Langeveld JP, Schreuder BE. Immunohistochemical evaluation of tonsillar tissue for preclinical screening of scrapie based on surveillance in Belgium. Vet Rec 1999;145:524-5.
46. O'Rourke KI, Baszler TV, Besser TE, Miller JM, Cutlip RC, Wells GA, et al. Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. J Clin Microbiol 2000;38:3254-9.
47. Schmerr MJ, Jenny AL, Bulgin MS, Miller JM, Hamir AN, Cutlip RC, et al. Use of capillary electrophoresis and fluorescent labeled peptides to detect the abnormal prion protein in the blood of animals that are infected with a transmissible spongiform encephalopathy. J Chromatog A 1999;853:207-14.
48. Brown P, Cervenáková L, Diringer H. Blood infectivity and the prospects for a diagnostic screening test in Creutzfeldt-Jakob disease. J Lab Clin Med. In press 2001.
49. Palsson PA. Rida (scrapie) in Iceland and its epidemiology. In: Prusiner SB, Hadlow WJ, editors. Slow transmissible diseases of the nervous system. Volume 1. New York: Academic Press; 1979. p. 357-66.
50. Brown P, Gadjusek DC. Survival of scrapie virus after 3 years' interment. Lancet 1991;337:269-70.

Appendix

Table A. Measures taken to prevent the spread of bovine spongiform encephalopathy to animals

Precautions	Great Britain[a]	European Union[a]	United States
BSE made a notifiable disease	June 1988	Apr 1990	Nov 1987
BSE surveillance, with histologic examination of brains	June 1988	May 1990	May 1990
Ban on ruminant protein in ruminant feed	July 1988		
Ban on export of UK cattle born before July 1988 feed ban		July 1989	
Ban on import of live ruminants and most ruminant products from all BSE countries			July/Nov 1989
Ban on export of UK cattle >6 months of age		Mar 1990	
Ban on SBO for use in animal nutrition; ban on export of SBO and feed containing SBO to EU countries	Sept 1990		
High-risk waste to be rendered at 133°C/3 bar/20 min (or other approved procedure)		Nov 1990	
Ban on export of SBO and feed containing SBO to non-EU countries	July 1991		
Ban on MBM from SBO in fertilizer	Nov 1991		
After Jan 1, 1995, rendering methods must sterilize BSE		June 1994	
Ban on mammalian MBM in ruminant feed		July 1994	
BSE surveillance includes immunohistologic features of brains			Oct 1993
Ban on mammalian protein in ruminant feed[b]	Nov 1994		Aug 1997
Ban on import of live ruminants and most ruminant products (including meat products) from all countries of Europe			Dec 1997
Immunologic testing for ruminant protein in animal feed		July 1995	
Mammalian MBM prohibited from all animal feed/fertilizer	Mar/Apr 1996		
Slaughtered cattle >30 months old (except certain beef cattle >42 months old) ruled unfit for animal use (hides for leather excluded)	Mar 1996		
Mammalian MBM and MBM-containing feed recalled	June 1996		
All mammalian waste to be rendered at 133°C/3 bar/20 min (or other approved procedure)		July 1996	
Cattle tracing system improved	Sept 1998		
Quarantine of 3 sheep flocks imported from Europe with possible exposure to BSE (4 animals die with atypical TSE)			Oct 1998
BSE surveillance of fallen stock (downer cows) is intensified			Oct 1998
Proposal to eradicate scrapie is rejuvenated			Nov 1999
Allow export of deboned beef from cattle >30 months old born after July 1996	Aug 1999		
Prohibit use of animal protein, including MBM and blood meal (but excluding milk, or fish meal for nonruminants) in feed for any farmed animal species (effective January 1, 2001)		Dec 2000	
Prohibit importation of rendered protein and rendering wastes originating or processed in Europe			Dec 2000

[a]In Northern Ireland and Scotland, dates of implementation sometimes differed from those shown for England and Wales; in addition, individual European Union countries often adopted different measures on different dates.
[b]Some exemptions, e.g., milk, blood, and gelatin.
BSE: bovine spongiform encephalopathy; EU = European Union; MBM = meat and bone meal (protein residue produced by rendering); SBO = specified bovine offals (brain, spinal cord, thymus, tonsil, spleen, and intestines from cattle >6 months of age); TSE = transmissible spongiform encephalopathy.

Table B. Measures taken to prevent the spread of bovine spongiform encephalopathy to humans

Precautions	Great Britain[a]	European Union[a]	United States
Compulsory slaughter of BSE-affected cattle	Aug 1988		
Destroy milk from affected cattle (except for milk fed to cows' own calves)	Dec 1988		
Ban on import of UK cattle born after July 1988 feed ban		July 1989	
Ban on SBO for domestic consumption	Nov 1989		
Ban on export to EU of SBO and certain other tissues, including lymph nodes, pituitaries, and serum	Apr 1990	Apr 1990	
Ban on export of live UK cattle (except calves <6 months old)	June 1990	June 1990	
Ban on use of head meat after skull opened	Mar 1992		
FDA recommends use of BSE/scrapie-free sources for materials used in dietary supplements; request for safety plans			Nov 1992
Cell lines used for biologicals should be BSE agent-free			May 1993
FDA requests that bovine source materials (except gelatin) used in manufacture of regulated products be restricted to BSE-free countries			Dec 1993
Bone-in beef only from farms with no BSE for 6 years; if not BSE-free, must be deboned with visible nervous and lymphatic tissue removed		July 1994	
FDA requests that bovine-derived materials for animal use or for cosmetics and dietary supplements not be sourced from BSE countries			Aug 1994
Thymus and intestines from calves <6 months old made SBO	Nov 1994		
Import of beef only from UK cattle 1) >30 months, or 2) from herds BSE-free for 6 years, or 3) if not BSE-free, deboned with visible nervous tissue and specified lymph nodes removed		July 1995	
SBO ban broadened to include whole skull (SBM)	Aug 1995		
MRM from bovine vertebral column banned and export prohibited	Dec 1995		
Removal of lymph nodes and visible nervous tissue from bovine meat >30 months exported to EU	Jan 1996		
Ban on export of all UK cattle and cattle products except milk		Mar 1996	
SBM ban broadened to include entire head (excluding uncontaminated tongue)	Mar 1996		
Slaughtered cattle >30 months (or certain beef cattle >42 months) ruled unfit for animal or human use (hides excepted)	Mar 1996		
FDA urges manufacturers of FDA-regulated human products to take steps to assure freedom from BSE agent		May 1996	
Partial lifting of export ban on tallow and gelatin		June 1996	
SBM ban broadened to include certain sheep and goat heads, spleens, and spinal cords (SRM)	Sept 1996		
FDA recommends withdrawal of plasma and plasma products made from pools to which persons who later died of CJD had contributed			Dec 1996
CNS tissues excluded from cosmetic products for use in EU		Jan 1997	
BSE cohort cattle in UK ordered slaughtered and destroyed	Jan 1997		
Proposed ban on SRM in cosmetics for use in EU (effective October 2000)		July 1997	
SBM controls for cosmetics and medicinal products	Mar 1997		
FDA request to manufacturers that no bovine gelatin from BSE countries be used in injectable, implantable, or ophthalmic products; and that special precautions be applied to gelatin for oral and topical use			Sept/Dec 1997
Ban on marketing cosmetic products containing SRM prepared before April 1, 1998		Mar 1998	
Allow export of beef and beef products from cattle >30 months in certified BSE-free herds from Northern Ireland		Mar 1998	
Importation of all plasma and plasma products for use in UK	Aug 1998		
FDA limits plasma product withdrawals to pools at risk for contamination by vCJD donors			Sept 1998
Slaughter and destruction of offspring born to BSE-affected cattle after July 1996	Jan 1999		
FDA guidance to defer blood donors with >6 months cumulative residence in UK during 1980-1996			Nov 1999
Leukodepletion of whole blood donations from UK residents	Jul/Nov 1999		
Public FDA discussion about possible risk associated with vaccines produced with bovine-derived materials from BSE countries			July 2000
Withdrawal and destruction of a potentially tainted 1989 lot of polio vaccine from one manufacturer	Oct 2000		
SRM ban implemented (effective October 2000)		July 2000	
Ban on slaughter techniques that could contaminate cattle carcasses with brain emboli (e.g., pithing or pneumatic stun guns), effective Jan 2001		July 2000	
All cattle >30 months old must have brain examinations for proteinase-resistant protein (PrP) before entering the food chain (effective Jan-Jun 2001)		Dec 2000	

[a]In Northern Ireland and Scotland, dates of implementation sometimes differed from those shown for England and Wales; in addition, individual EU countries often adopted different measures on different dates.
CNS = central nervous system; EU = European Union; MRM = mechanically recovered meat; SBM = specified bovine materials (SBO plus entire head, including eyes but excluding tongue); SBO = Specified bovine offals (brain, spinal cord, thymus, tonsils, spleen, and intestines from cattle >6 months old); SRM = specified risk materials (SBM plus sheep and goat heads and spleens from animals of any age, and spinal cords from animals >1 year old).

Pesticides and Public Health: Integrated Methods of Mosquito Management

Robert I. Rose
U.S. Environmental Protection Agency, Washington, DC, USA

Pesticides have a role in public health as part of sustainable integrated mosquito management. Other components of such management include surveillance, source reduction or prevention, biological control, repellents, traps, and pesticide-resistance management. We assess the future use of mosquito control pesticides in view of niche markets, incentives for new product development, Environmental Protection Agency registration, the Food Quality Protection Act, and improved pest management strategies for mosquito control.

Vector-borne diseases (including a number that are mosquito-borne) are a major public health problem internationally. In the United States, dengue and malaria are frequently brought back from tropical and subtropical countries by travelers or migrant laborers, and autochthonous transmission of malaria and dengue occasionally occurs. In 1998, 90 confirmed cases of dengue and 1,611 cases of malaria were reported in the USA (1) and dengue transmission has occurred in Texas (2). Other vector-borne diseases continue to pose a public health threat. Even though the reported incidence of most of these diseases is low (in 1997, 10 cases of eastern equine encephalitis, 115 of LaCrosse, and 14 of St. Louis encephalitis [SLE]), occasional epidemics, e.g., of SLE (1,967 cases in 1975 and 247 cases in 1990, mostly in Florida [3]) have resulted in aerial applications of insecticides, primarily malathion. In addition, new vector-borne threats continue to emerge. In 1999, West Nile virus, an Old World flavivirus related to Saint Louis encephalitis virus, was first recorded in New York (4). The virus, which is transmitted by anthropophilic mosquitoes, caused a serious outbreak (62 cases, 7 deaths) and signaled the potential for similar outbreaks in the Western Hemisphere. Pesticides, which traditionally have been used in response to epidemics, have a role in public health as part of sustainable integrated mosquito management for the prevention of vector-borne diseases. We assess the future use of pesticides in view of existing niche markets, incentives for new product development, Environment Protection Agency (EPA) registration, the Food Quality Protection Act (FQPA), and improved pest management strategies for mosquito control.

Address for correspondence: Robert I. Rose, USDA, APHIS, PPQ, Unit 147, 4700 River Road, Riverdale, MD 20737, USA; fax: 301-734-8669; e-mail: Bob.I.Rose@usda.gov.

Sustainable Integrated Mosquito Management and Public Health

Mosquito control in the United States has evolved from reliance on insecticide application for control of adult mosquitoes (adulticide) to integrated pest management programs that include surveillance, source reduction, larvicide, and biological control, as well as public relations and education. The major principles of integrated mosquito management are available at a new Public Health Pest Control Manual internet website (5). Adulticides still play a vital role when flooding causes extreme numbers of nuisance mosquitoes or when outbreaks of diseases such as SLE occur.

Surveillance programs track diseases harbored by wild birds and sentinel chicken flocks; vector-borne pathogens in mosquitoes; adult and larval mosquitoes and larval habitats (by aerial photographs, topographic maps); mosquito traps; biting counts; and follow-up on complaints and reports by the public. When established mosquito larval and adult threshold populations are

exceeded, control activities are initiated. Seasonal records are kept in concurrence with weather data to predict seasonal mosquito larval occurrence and adult flights.

Source reduction consists of elimination of larval habitats or rendering of such habitats unsuitable for larval development. Public education is an important component of source reduction. Many county or state mosquito control agencies have public school education programs that teach children what they and their families can do to prevent mosquito proliferation. Other forms of source reduction include open marsh water management, in which mosquito-producing areas on the marsh are connected by shallow ditches to deep water habitats to allow drainage or fish access; and rotational impoundment management, in which the marsh is minimally flooded during summer but is flap-gated to reintegrate impoundments to the estuary for the rest of the year.

Biological control includes use of many predators (dragonfly nymphs and other indigenous aquatic invertebrate predators such as *Toxorhynchites* spp. predacious mosquitoes) that eat larvae and pupae; however, the most commonly used biological control adjuncts are mosquito fish, *Gambusia affinis* and *G. holbrooki.* Naturally occurring *Fundulus* spp. and possibly *Rivulus* spp., killifish, also play an important role in mosquito control in open marsh water management and rotational impoundment management. Like many fish, mosquito fish are indiscriminate feeders that may eat tadpoles, zooplankton, aquatic insects, and other fish eggs and fry (6). However, since they are easily reared, they have become the most common supplemental biological control agent used in mosquito control. The entomopathogenic fungus, *Laginidium giganteum,* has been registered for mosquito control by EPA under the trade name Liginex, but products have not become readily available. The pathogenic protozoon, *Nosema algerae,* has also not become available for technical reasons. Entomoparasitic nematodes such as *Romanomermis culicivorax* and *R. iyengari* are effective and do not require EPA registration but are not easily produced and have storage viability limitations. A predacious copepod, *Mesocyclops longisetus,* preys on mosquito larvae and is a candidate for local rearing with *Paramecium* spp. for food.

Mosquito traps (such as the New Jersey and the Centers for Disease Control and Prevention designs) have been used for monitoring mosquito populations for years. New designs using mechanical control to capture adult mosquitoes have now become available. These designs use compressed carbon dioxide, burning propane, and octenol to attract mosquitoes and fans to control air flow. The new technology is expensive: these traps may cost well over $1,000 each. Electric high-voltage insect traps (“bug zappers”) with “black” or ultraviolet light sources do not provide satisfactory adult mosquito control and kill insects indiscriminately.

Pesticides

Pesticides used by state or local agencies to control nuisance or public health pests have warning labels and directions to minimize risks to human health and the environment. These pesticides are applied by public health employees who are specifically trained to follow proper safety precautions and directions for use. State or local mosquito control programs are funded by taxes and subject to public scrutiny. The environmental hazards precautionary statements on many mosquito insecticide labels state that insecticides are toxic to birds, fish, wildlife, aquatic invertebrates, and honeybees. Because of the low rates of application used to control mosquitoes and the special public health pest control training of most applicators, hazard to nontargeted organisms is limited. However, honeybees may be killed if exposed when foraging, so proper precautions are warranted. Human exposure in residential areas is also uncommon because of the very low application rates, ultra low-volume methods (ULV), treatment at night when people are indoors, pesticide applicator training, and public prenotification before application. Pesticide applicators who mix, load, and apply the concentrated insecticides use personal protective equipment to avoid exposure and closed systems to pump insecticides from storage to spray equipment.

The Federal Food Drug and Cosmetic Act (FFDCA) 21 USC 9§406 is the regulation that limits the quantity of any poisonous or deleterious substance added to food. A pesticide residue is the pesticide or its metabolites in or on raw agricultural commodities or processed food and feed. A tolerance is the maximum limit of a pesticide residue considered safe. Tolerances are relevant to adult mosquito control because wind drift may carry the pesticide over agricultural

crops where residues subject to legal tolerance requirements may occur. Crop tolerances are listed in the Code of Federal Regulations (7).

Larvicides

Detection of large numbers of immature mosquitoes in areas where source reduction or biological control is not feasible may require larvicide treatment to prevent the emergence of adult mosquitoes. Use of larvicides is less controversial than use of adulticides, although use of larvicides may lead to public concern about their effects on untargeted beneficial aquatic arthropods and vertebrates (Table).

Adulticides

Effective sustainable integrated mosquito management programs strive to prevent large

Table. Pesticides used for mosquito control in the United States

Name	Trade name	Formulation	Application	Advantage	Limitation
Temephos	Abate	G, EC	Larvae	Usually lowest cost	Nontarget effects, some resistance
Methoprene	Altosid	G, B, P, LC	Larvae	Residual briquets, non-target safety	Cannot be certain of performance until too late to retreat
Oils	BVA, Golden Bear	Oil	Larvae, pupae	Acts on pupae	Oil film, subsurface larvae
Monomolecular film	Agnique	Liquid	Larvae, pupae	Acts on pupae	Subsurface larvae
Bacillus thuringiensis israelensis (Bti)	Aquabac, Bactimos, LarvX, Teknar, Dunks	WDG, AS, P,G,B	Larvae	Nontarget safety, Briquets control 30+ days	Short window of treatment opportunity. pupae
Bacillus sphaericus (Bs)	VectoLex	G, WDG	Larvae	Nontarget safety	Pupae, only works in fresh water
Malathion	Fyfanon, Atrapa, Prentox	ULV, thermal fog	Adults	Tolerances	OP, some resistance
Naled	Dibrom, Trumpet	ULV, EC, thermal fog	Adults	Tolerances	OP, corrosive
Fenthion	Batex	ULV	Adults	None specified	OP, Florida only, RUP, tolerances
Permethrin	Permanone, AquaResilin, Biomist, Mosquito-Beater	ULV, thermal fog, clothing treatment	Adults, clothing treatment for ticks and mosquitoes	Low vertebrate toxicity	None specified
Resmethrin	Scourge	ULV, thermal fog	Adults	Low vertebrate toxicity	RUP, no tolerance for residue on crops
Sumithrin	Anvil	ULV, thermal fog	Adults	Low vertebrate toxicity	No tolerance
Pyrethrins	Pyrenone, Pyronyl	ULV, EC	Adults, larvae	Natural pyrethrum, tolerances	May be costly

AS = Aqueous Suspension; B = Briquets; EC = Emulsifiable Concentrate; G = Granules; LC = Liquid Concentrate; P = Pellets; ULV = Ultra Low Volume; WDG = Water-Dispersible Granule; OP = Organophospate insecticide; RUP = Restricted Use Product

flights or swarms of mosquitoes through all the measures described above, but heavy precipitation, flooding, high tides, environmental constraints, inaccessible larval habitats, missed breeding sites, human disease outbreaks, as well as budget shortfalls, absent employees, or equipment failures, may necessitate use of adulticides (Table). Some local mosquito control programs would use an integrated program if they had adequate resources, but may be so limited in funding and personnel that adulticiding trucks are the only means of mosquito intervention.

Effective adult mosquito control with insecticides requires small droplets that drift through areas where mosquitoes are flying. The droplets that impinge on mosquitoes provide the contact activity necessary to kill them. Large droplets that settle on the ground or vegetation without contacting mosquitoes waste material and may cause undesirable effects on nontargeted organisms. To achieve small droplets, special aerial and ground application ULV equipment is used. Insecticides are applied in a concentrated form or technical grade and at very low volumes such as 1 oz (29.6 mL) per acre. Typically, aerial applications produce spray droplets of 30 to 50 microns measured as mass median diameter, with $\leq$2.5% of the droplets exceeding 100 microns. Ground ULV applicators produce droplets of 8 to 30 microns, with none >50 microns mass median diameter. Large droplets of malathion, naled, and fenthion in excess of 50 to 100 microns can damage automotive or similar paint finishes.

Adulticide applications, particularly aerial applications and thermal fogging, are quite visible and contribute to public apprehension. Ground ULV application may be less alarming than aerial application but is not effective over large or inaccessible areas. Preferable air currents for ground applications are 3.2 kph to 12.9 kph and not in excess of 16.1 kph. Excessive wind and updrafts reduce control, but light wind is necessary for drifting spray droplets. With insecticide application by air using high-pressure pumps of 2,500 lbs psi, special nozzles, proper aerial application altitude and wind drift, mosquito control is achievable for several miles downwind with minimal spray deposit below the aircraft, as a result of improved atomization of the insecticide. This technology is being developed and needs validation under different conditions with different mosquito species before it can be universally used. Thermal fogging, which was commonly used before ULV applications became prevalent, continues to be used in a few areas in the United States and is still widely used in other countries. The insecticide is diluted with petroleum oil and vaporized with heat into a dense, highly visible fog of very small uniform droplets, which allows tracking the plume downwind to target areas. Although this fog reduces visibility, it may also penetrate vegetation better than a ULV application. Small electric or propane thermal foggers are available for consumer use in retail stores at a cost of approximately $60.00.

Adult mosquitoes are easily controlled with insecticides applied at extremely low rates. For example, malathion is applied at 3 fl oz per acre (219.8 mL/ha) for mosquitoes, while the rate for agriculture is as much as 16 fl oz per acre (1,172 mL/ha).

Insecticide Resistance

Vector resistance to certain larvicides and adulticides has occurred periodically. Failure of mosquito control indicating resistance must be verified by laboratory analysis or use of test kits because other factors (improper equipment calibration, dilution, timing and other application errors, off-specification products, climatic factors) can prevent insecticides from providing satisfactory control in the field. Resistance may occur between insecticides within a class or could be passed from immature to adult stages subject to the same insecticidal mode of action. Additionally, different species of mosquitoes may inherently vary in susceptibility to different larvicides and adulticides. Insecticides with different modes of action can be alternated to prevent resistance. Even though source reduction and use of predators such as larvivorous fish are also used for sustainable integrated mosquito management, only two chemical classes of adulticides (organophosphates and pyrethroids) with different modes of action are available. Biological controls (including birds and bats) may be present, but often not in sufficient numbers to provide satisfactory alternative control, particularly in coastal areas where salt-marsh mosquitoes are abundant or when human disease outbreaks occur. Therefore, sustained integrated mosquito management requires alternative use of different classes of insecticides, in conjunction

with resistance monitoring, source reduction, biological control, and public education.

Repellents

Insect repellents, primarily N,N-diethylmetatoluamide (DEET), are used to prevent nuisance bites from mosquitoes (as well as ticks, biting flies, and mites) and may aid in lowering disease transmission from these pests. However, they should not be relied upon to prevent disease transmission, particularly where Lyme disease or encephalitis are endemic or malaria, yellow fever, or other vector-borne diseases are prevalent. Repellents, mosquito coils, and permethrin clothing treatment products are subject to EPA pesticide registration performance requirements (8). Information on safe use of repellents is located at the EPA Office of Pesticide Programs website (9). Citronella and its oil for mosquitoes and 30 other active ingredients are exempted from EPA pesticide registration (10). However, some of these products may not be efficacious.

Future of Public Health Pesticides

The past decade has seen a sharp rise in public apprehension concerning the use of pesticides, although state and federal regulations are well established for the assessment and mitigation of their human and environmental risks. Response to public concern over safety of pesticides prompted the FQPA, which includes provisions to protect availability of public health pesticides. However, public health pesticides are in jeopardy for the following reasons: In the United States, mosquito control programs are often for nuisance rather than disease vector control and not many insecticides are registered for this use. None of the mosquito adulticides commonly used were developed recently; their registrations are up to 44 years old. Mosquito control is only a niche market compared with agricultural pest control, which includes pesticides for use on corn, soybeans, and cotton, as well as the high-profit home, garden, and structural pest control markets. As pesticide companies have merged to form multinational conglomerates, the most profitable markets are those that drive corporate decisions. At present, it may require $50 million or more to develop and register a new pesticide with EPA. Furthermore, several years of the patent life elapse before costs are recouped and profits accrue.

Vector control uses of existing pesticides, particularly adulticides, often follow agricultural registration and commercialization as a means of expanding sales into new markets. Performance data are not usually required for registration of agricultural pesticides, but these data are required for registration of public health pesticides. For mosquito control, these data are often obtained under an experimental use permit, which requires application to EPA, submission or reference to a portion of the pesticide registration requirements according to CFR 40 § 158 Data Requirements for Registration and Reporting (7,8). Testing for mosquito adulticides or larvicides is typically done by universities and mosquito control or abatement districts, although it may be done by companies or state or federal research organizations, such as the Department of Defense or the U.S. Department of Agriculture. In addition to defining dose rates, formulations, environmental variables, and effects that must be accommodated, testing under an experimental use permit provides a means of market introduction through user and customer experience, presentations at professional society meetings, and journal publications.

Pesticide marketing often involves distributors or dealers who specialize in the market if the manufacturers do not deal directly. Profit margins that add to price are required by distribution chains. Public agencies solicit competitive bids for pesticides, which squeeze margins further, thus affecting marketing incentives. Mosquito adulticides are used at very low rates of active ingredient per acre, which limits sales volumes and margins. Some seasons have few mosquitoes, so sales are low. Product liability also plays an important role in reducing incentives because of possible personal and class-action lawsuits or court injunctions against pesticides applied over populated areas.

The Federal Insecticide, Fungicide, and Rodenticide Act and FQPA

The Federal Insecticide, Fungicide, and Rodenticide Act 7 USC 136 and FFDCA were amended by the FQPA of 1996. Amendments pertinent to mosquito nuisance and vector control include the following: review of a pesticide's registration every 15 years; expediting minor use registrations; special provisions for public health pesticides; aggregate (all modes of

exposure from a single pesticide) and cumulative (all pesticides with the same mode of action) risk assessments; an additional safety factor of up to 10 X for children; collection of pesticide use information; and integrated pest management. Special provisions for public health pesticides include the following: risks and benefits considered separately from those of other pesticides; exemption from fees under certain circumstances; development and implementation of programs to control public health pests; Department of Health and Human Services (DHHS)-supported studies required for reregistration when needed; and appropriations of $12 million for the first year after enactment and similar funding as needed in succeeding years to carry out public health pesticide provisions of the Act. The Act describes a consultation process between EPA and DHHS before any public health pesticide registration is suspended or canceled and allows additional time for submission of data. The first group of pesticides under review are the organophosphate cholinesterase inhibitors, including temephos, fenthion, naled, chlorpyrifos, and malathion. Should risk assessments result in detection of risk of concern to the Agency, cancellation or mitigations of use may follow, as exemplified by recent chlorpyrifos and diazinon use cancellations. Risk assessments may be based on data from acute and chronic toxicology and exposure studies, models that simulate exposure scenarios, reports of adverse incidents to humans and wildlife, extrapolation, maximum label use rate assumption, and worst-case exposure scenarios.

Even though the FQPA provisions were intended by Congress to ensure that existing public health pesticide uses are not lost without economically effective alternatives, the provisions may not be adequate. If FQPA results in cancellation of major agricultural uses of a pesticide that is also used in public health, it may become no longer profitable for the manufacturer to produce small quantities for mosquito control, thus ending production of the pesticide. Since adulticides used for mosquito control were registered decades ago, the data supporting their registrations may be insufficient to meet current requirements. The substantial cost involved in updating the data required for reregistration will have to be paid by pesticide registrants or the Federal government though the authorized and appropriated funding in FQPA. Data to support reregistration done at public expense are not proprietary. Registrants need proprietary data to protect their market shares from generic pesticide competition from overseas manufacturers that can use public data to support their own registrations; therefore, they may not consider requesting public funds to pay for new data to support existing registrations. However, if generic safety studies applicable to several public health pesticides are required by EPA for all reregistrations, the data could be generated by a task force of registrants and county, state, and Federal public health agencies, which would then request public funding under the provisions of the Act.

Although the development of new mosquito insecticides, particularly adulticides, is not expected to accelerate in the near future, integrated pest management tools and techniques should improve as a result of FQPA funding and the need to control continued vector-borne disease outbreaks. Integrated pest management tools have strengths and weaknesses, and continued availability of adulticides is critical. Therefore, implementation of the public health pesticide provisions of FQPA must include substantial comparative risk-benefit analyses of the significance of vector-borne disease impacts versus potential human and environmental toxic effects of pesticides used to control public health pests, both in the USA and other countries affected by EPA pesticide regulatory decisions. Public information and legislative campaigns have also become necessary to preserve the availability and use of pesticides for disease vector control as FQPA has been implemented and with the concurrent spread of West Nile virus.

Dr. Rose is an arthropod biotechnologist with the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture.

References

1. Centers for Disease Control and Prevention. Summary of notifiable diseases, United States, 1998. MMWR Morb Mortal Wkly Rep 1999:47:1-93. Available from: URL: http.cdc.gov/mmwr//preview/mmwrhtml/mm4753a1.htm
2. Rawlings JA, Hendricks KA, Burgess CR, Campman RM, Clark GG, Tabony LJ, Patterson MA. Dengue surveillance in Texas, 1995. Am J Trop Med Hyg 1998;59:95-9.

3. Centers for Disease Control and Prevention. Confirmed arboviral encephalitis cases reported to CDC by state health departments, by type and state, United States 1964-1997. Available from: URL: http://www.cdc.gov/ncidod/dvbid/arbor/arbocase.htm
4. Rappole JH, Derrickson SR, Hubálek Z. Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerg Infect Dis 2000;6:319-28.
5. University of Florida Entomology and Nematology Department. Public health pest control manual, the national USA manual and related information. Available from: URL: http://www.ifas.ufl.edu/~pest/vector
6. Courtenay WR, Meffe, GK. Small fishes in strange places; a review of introduced poeciliids. In: Meffe GK, Snelson EF, editors. Ecology and evolution of live-bearing fishes (Poeciliidae). New Jersey: Prentice Hall; 1989. p. 319-31.
7. Protection of the Environment, 40 C.F.R. Parts 150 to 180, July 2000 revision. Available from: URL: http://www.access.gpo.gov/nara/cfr/cfr-table-search.html
8. U.S. Environmental Protection Agency. Product Performance Test Guidelines, OPPTS 810.3700, Insect Repellents for Human Skin and Outdoor Premises. Washington: U.S. Government Printing Office; 1999. Available from: URL: http://www.epa.gov/OPPTS_Harmonized
9. U.S. Environmental Protection Agency. Using insect repellents safely. EPA Office of Pesticide Programs. Available from: URL: http://www.epa.gov/pesticides/citizens/insectrp.htm
10. U.S. Environmental Protection Agency. Notice to Manufacturers, Formulators, Producers and Registrants of Pesticide Products on Minimum Risk Pesticides Exempted under FIFRA Section 25(b) Pesticide Registration (PR). Notice 2000-6, 2000. Labeling Unit, Registration Division (7505C). Washington: Office of Pesticide Programs, EPA; 2000. Available from: URL: http://www.epa.gov/opppmsd1/PR_Notices/#2000

Quinolone and Macrolide Resistance in *Campylobacter jejuni* and *C. coli*: Resistance Mechanisms and Trends in Human Isolates

Jørgen Engberg,* Frank M. Aarestrup,† Diane E. Taylor,‡ Peter Gerner-Smidt,* and Irving Nachamkin§
*Statens Serum Institut, Copenhagen, Denmark; †Danish Veterinary Laboratory, Copenhagen, Denmark; ‡ University of Alberta, Edmonton, Alberta, Canada; §University of Pennsylvania, Philadelphia, Pennsylvania, USA

The incidence of human *Campylobacter jejuni* and *C. coli* infections has increased markedly in many parts of the world in the last decade as has the number of quinolone-resistant and, to a lesser extent, macrolide-resistant *Campylobacter* strains causing infections. We review macrolide and quinolone resistance in *Campylobacter* and track resistance trends in human clinical isolates in relation to use of these agents in food animals. Susceptibility data suggest that erythromycin and other macrolides should remain the drugs of choice in most regions, with systematic surveillance and control measures maintained, but fluoroquinolones may now be of limited use in the empiric treatment of *Campylobacter* infections in many regions.

Campylobacter jejuni subsp. *jejuni* (*C. jejuni*) and *C. coli* have been recognized since the late 1970s as important agents of gastrointestinal infections throughout the world; in the United States, these infections affect approximately 1% of the population each year (1). Contaminated food is the usual source of human infections; therefore, the presence of fluoroquinolone- and macrolide-resistant strains in the food chain has raised concerns that the treatment of human infections will be compromised. Most cases of *Campylobacter* enteritis do not require antimicrobial treatment, being brief, clinically mild, and self-limiting (2-4). However, a substantial proportion of these infections require treatment. These include severe and prolonged cases of enteritis, septicemia, and other extraintestinal infections. Erythromycin has been the most commonly used agent for treating *Campylobacter* enteritis (2,5).

In the 1980s, the introduction of fluoroquinolones, which are effective against most major pathogens causing bacterial enteritis, offered a new approach to antibiotic intervention (6). Fluoroquinolones initially had good in vitro activity for thermophilic *Campylobacter* species, as well as for members of the family of Enterobacteriaceae.

Early clinical trials of both community-acquired acute diarrhea and traveler's diarrhea caused by *Campylobacter* demonstrated that patients treated with a fluoroquinolone had good clinical response (6,7). It soon became apparent, however, that resistance in *Campylobacter* spp. could arise in vivo, sometimes after only one or two administrations of fluoroquinolones (8). Moreover, Endtz and colleagues (9) reported as early as 1991 that the emergence of quinolone-resistant *C. jejuni* and *C. coli* isolated from humans in the Netherlands coincided with the introduction of fluoroquinolones in veterinary medicine.

Fluoroquinolone resistance in *Campylobacter* from food animals is now recognized as an emerging public health problem. Smith et al. from Minnesota (10) found that patients infected with resistant *C. jejuni* had longer duration of

Address for correspondence: Jørgen Engberg, Department of Gastrointestinal Infections, Division of Diagnostics, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark; fax: 45-3268-8238; e-mail: eng@ssi.dk.

diarrhea than patients with fluoroquinolone-sensitive isolates. As *Campylobacter* infections can be serious in immunocompromised patients, the identified treatment failure raises the concern that fluoroquinolone-resistant strains may increase *Campylobacter*-associated deaths in this group of patients.

Mechanism of Macrolide Resistance in *Campylobacter*

Erythromycin binds to the ribosome but, unlike larger macrolides, appears to cause dissociation of the peptidyl-tRNA, rather than blocking the peptidyltransferase activity (11).

In *C. jejuni* and *C. coli*, erythromycin resistance is chromosomally mediated and is due to alteration of the ribosome (12); the resistance mechanism is not consistent with presence of an rRNA methylase, modification of the antibiotic, or efflux (13). Whole ribosomes or 50S subunits were purified from erythromycin-resistant strains and shown to bind much less erythromycin than ribosomes from sensitive strains. In a closely related bacterium, *Helicobacter pylori*, resistance to clarithromycin is due to an alteration of one of two adenine residues in the 23S rRNA at the erythromycin-binding site (14). Sequencing of the 23S rRNA genes from erythromycin-resistant *Campylobacter* spp. identified mutations at these same sites, which are most probably responsible for resistance (Figure 1) (15).

Mechanism of Fluoroquinolone Resistance in *Campylobacter*

Fluoroquinolone resistance in *C. jejuni* appears to be due most often to mutations in the genes encoding subunits of DNA gyrase (*gyr*A) and only occasionally to topoisomerase IV (*par*C) (Figure 1). DNA gyrase purified from quinolone-resistant mutants of *C. jejuni* was 100-fold less sensitive to inhibition by quinolones than the wildtype gyrase (19). Cloning and sequencing of the *C. jejuni gyr*A gene demonstrate that mutations in *gyr*A at positions Thr-86, Asp-90, and Ala-70 were responsible for resistance (16,17). Mutations at Thr-86 are associated with higher level resistance to nalidixic acid (MIC 64-128 µg/mL) and ciprofloxacin (MIC 16-64 µg/mL) than mutations at Asp-90 or Ala-70. *C. jejuni* isolates resistant to even higher levels of quinolones (ciprofloxacin MIC of 125 µg/mL) carry two mutations, one in *gyr*A Thr-86 and the other in the topoisomerase IV subunit *par*C at Arg-139 (18).

Resistance	**Mutation**
Macrolide →	Domain V of 23S rRNA A2058 → G A2059 → G
Fluoroquinolone →	*gyra* → Thr - 86 (higher MIC) Thr - 90 Ala - 70 (lower MIC)
→	*gyra* → Thr - 86 + *parc*→ Arg - 139 (highest MIC)

Figure 1. Macrolide and fluoroquinolone resistance mechanisms reported in *Campylobacter* species. For macrolide resistance, mutations are at either position shown (*Escherichia coli* coordinates) in up to all three copies of ribosomal RNA (14,15, and CA Trieber & DE Taylor, unpub. data). Fluoroquinolone resistance depends on a mutation in the quinolone resistance determining region of DNA gyrase A (*Gyr*A). For typical MICs see text and references 16-18. The strains with highest resistance levels had mutations in both *Gyr*A and topoisomerase IV *Par*C.

Evidence of efflux of fluoroquinolones in *C. jejuni* (20) also exists. Passage of the bacteria on pefloxacin-containing agar has led to the isolation of a fluoroquinolone-resistant strain. This strain was also resistant to tetracycline, erythromycin, chloramphenicol, and several β-lactams. The pefloxacin-resistant strain carried a mutation at Thr-86 of *gyr*A, likely responsible, in part, for fluoroquinolone resistance. Broad-specificity efflux pumps in *C. jejuni,* which cause fluoroquinolone resistance, have not yet been shown to be clinically relevant.

Use of Macrolides and Quinolones in Food Animals

Antibiotics of the macrolide-lincosamide group have been used in treating food animals worldwide for several decades. The most commonly used agents have been lincomycin and tylosin for controlling dysentery and *Mycoplasma* infections in swine and spiramycin for treating mastitis in cattle. For the past 20 years, tylosin has also been the most commonly used agent for growth promotion in swine production worldwide, whereas spiramycin has been

commonly used in poultry. The use of macrolides for growth promotion has been banned in all European Union countries since July 1999.

Several fluoroquinolones are available for treating food animals, such as poultry, cattle, pigs, and fish, in many countries. While information on global use is limited, worldwide use in food animals was estimated at 120 tons in 1997; use in humans has been estimated at more than 800 tons (21). Data are available only for the year of veterinary licensing of fluoroquinolones by country (Table 1). Licensing for use does not necessarily mean that the drug is actually used, so even these data have to be considered with caution. However, quinolone treatment of *Campylobacter*-colonized broiler chickens has induced quinolone resistance under experimental conditions (23).

Macrolide and Quinolone Resistance in Isolates from Food Animals and Foods of Animal Origin

Campylobacter is carried in the intestinal tract of wild and domestic animals and, as result

Table 1. Veterinary licensing of fluoroquinolones in selected countries

Country	Substance	Licensing year	Animal species
Austria (22)	Enrofloxacin	1992	Cattle, pigs, poultry
	Danofloxacin	1996	Poultry
	Difloxacin	1998	Poultry
Canada[a]	Enrofloxacin	1987 (withdrawn in 1997)	Turkey (egg dip)
Denmark (22)	Enrofloxacin	1991	Cattle, pigs, poultry
	Danofloxacin	1993	Poultry
	Difloxacin	1998	Poultry, turkey
	Marbofloxacin	1998	Cattle, pigs, dogs, cats
Finland (22)	Enrofloxacin	1992 (oral use withdrawn in 1999)	Pigs
	Difloxacin	1998	Poultry
France (22)	Enrofloxacin	1991	Cattle, poultry
	Danofloxacin	1996	Cattle
	Marbofloxacin	1993	Cattle
	Difloxacin	1998	Poultry
Italy (22)	Enrofloxacin	1989	Cattle, pigs, poultry
	Difloxacin	1998	Poultry
Japan[b]	Enrofloxacin	1991	Cattle, poultry
		1992	Pigs
	Danofloxacin	1992	Poultry
		1993	Cattle, pigs
	Ofloxacin	1992	Poultry
	Orbifloxacin	1993	Cattle, pigs
	Difloxacin	1996	Pigs
	Norfloxacin	1998	Poultry
Netherlands (22)	Enrofloxacin	1987	Cattle, pigs, poultry
	Difloxacin	1998	Poultry
Spain (22)	Enrofloxacin	1986	Cattle, pigs, poultry
	Difloxacin	1998	Poultry
United Kingdom (22)	Enrofloxacin	1993	Cattle, pigs, poultry
	Danofloxacin	1993	Poultry
	Marbofloxacin	1995	Cattle
	Difloxacin	1998	Poultry
USA[c]	Enrofloxacin	Approx. 1987-88	Dogs, cats
		1996	Poultry
	Sarafloxacin	1999	Cattle
		1995	Poultry

[a]RJ Irwin, Health Canada, 1999. pers. comm.
[b]Y Tamura, National Veterinary Assay Laboratory, Japan, 1999. pers. comm.
[c]JL Watts. Pharmacia/Upjohn, Kalamazoo, Michigan, 1999. pers. comm.

of fecal contact during processing, frequently contaminates foods derived from animals. *C. jejuni* is predominant in broilers and cattle but is infrequent in pigs (where *C. coli* predominates) (24). In food animals, the prevalence of resistance to erythromycin is generally higher in *C. coli,* in particular in *C. coli* isolates from pigs, than in *C. jejuni* (24-26). In a recent study from Spain (27), rates of erythromycin and quinolone resistance in *C. coli* from pigs were 81% and 100%, respectively. High erythromycin resistance in pigs may be related to extensive veterinary use of macrolides (5,28).

In food products of animal origin, the occurrence of *Campylobacter* is much higher in poultry than in other categories, e.g., pork or beef (29). Therefore, *Campylobacter* resistance data are primarily based on poultry products, especially broiler meat. For a number of countries, fluoroquinolone-resistance rates are similar in isolates from poultry products and humans (10,25,27,30-32). In the United Kingdom, enrofloxacin (a derivative of ciprofloxacin) was first licensed in late 1993; before then, domestically bred chickens were less frequently infected with quinolone-resistant campylobacters than imported chicken products. Using a simple model, researchers were able to correlate the previously observed resistance percentage in domestically acquired cases with estimates of the amount of imported chicken consumed in the United Kingdom (32). In recent data from Spain and Taiwan, rates of erythromycin resistance were 17% and 17%, respectively, in *C. jejuni* isolated from foods, whereas for *C. coli* the figures were 50% and 83%, respectively (27,31).

Transmission of Resistant *Campylobacter* from Animals to Humans

Campylobacteriosis is primarily a zoonosis. Evidence to indicate that fresh raw meat, especially poultry, is a major source of infection is ample, even though other sources such as raw milk, water, and pets may contribute to human infection (1,5,33-38).

Studying the transmission of antimicrobial resistance from animals (especially poultry to humans) has been difficult because the chain of transmission is often complex. The number of macrolide- and fluoroquinolone-resistant isolates from humans is influenced by several factors including veterinary use of macrolides (approved for use as antimicrobial growth promoters or as therapeutic drugs) and fluoroquinolones (only approved as therapeutic drugs) at a given location (24,39); association with recent or current antimicrobial treatment of patients; the origin of isolates (children vs. adults; inpatients vs. outpatients); travel (10,40-45); and sampling strategy and susceptibility testing procedures (no consensus as to method, media, culture conditions, or breakpoints [43,46]). These factors stress the need for cautious interpretation and comparison of data from different centers. However, several studies have shown that food animals can be a substantial source of infection in humans and that the same serotypes and genotypes can be isolated from humans and food animals (29,36,37,47-49). DNA profiling of Danish *C. jejuni* serotype O:2 strains using pulsed-field gel electrophoresis with four restriction enzymes identified common genotypes in humans, poultry, cattle and swine (SLW On, EM Nielsen, and J Engberg, unpub. data). Typing data on resistant isolates is sparse, but Smith and colleagues (10) found DNA fingerprints of quinolone-resistant *C. jejuni* from U.S.-produced poultry identical to those of resistant *C. jejuni* from domestically acquired infections in humans. Therefore, the susceptibility of humans strains originating in animals to antibiotics can be related to the exposure of animal strains to antibiotic agents used in farming.

Is There a Link Between Macrolide and Fluoroquinolone Use in Humans and Resistant *Campylobacter* Infections?

Some investigators suggest that resistance in *C. jejuni* and *C. coli* is driven by use of antibiotics for treating human infections rather than by veterinary use. Induction of macrolide resistance during treatment has been reported infrequently (50). However, induction of macrolide resistance may play a role in areas with a large reservoir of asymptomatic *Campylobacter* carriers and frequent use of macrolides in humans.

Induction of fluoroquinolone resistance during treatment is well recognized and often reported (8,51-53). A predicted 10% of patients treated with a fluoroquinolone for *Campylobacter* enteritis harbor quinolone-resistant *Campylobacter* strains (6). Recently, Ellis-Pegler (53) found that fluoroquinolone resistance developed in 18% to 28% of patients in their prospective trial. Development of resistance has been

reported within 24 hrs of treatment, but prolonged therapy, e.g., in immunosuppressed patients, is also a risk factor (52,54).

Smith et al. (10) showed that use of a quinolone before culture accounted for a maximum of 15% of resistant isolates during 1996 to 1998. In addition, an increasing number of reports claim that fluoroquinolone-resistant strains have been isolated from patients who had not received medical treatment, suggesting that strains were already fluoroquinolone resistant before causing the infection (7,31,32,55-57). Since human-to-human transmission of *C. jejuni* and *C. coli* is rare (9), patients infected with resistant *Campylobacter* are not an important source of resistant *Campylobacter* for other humans.

Before fluoroquinolones were introduced in veterinary medicine, they were widely used in human medicine in a number of countries, including the Netherlands and the United States (since 1985 and 1987, respectively), without emergence of quinolone resistance in *Campylobacter* in humans. In contrast, emerging quinolone resistance in humans often coincides with or follows the approval of fluoroquinolones in animal husbandry (Table 1, Figure 2). Thus, while human macrolide and fluoroquinolone use contributes to the increase in resistance in humans, their relative contribution to increase in resistance compared to the use of these agents in husbandry appears to be small.

Frequency of Macrolide Resistance in Human Isolates

Data on erythromycin and azithromycin resistance in *C. jejuni*, *C. coli,* and the two organisms combined, isolated from humans around the world since 1989, differ by country

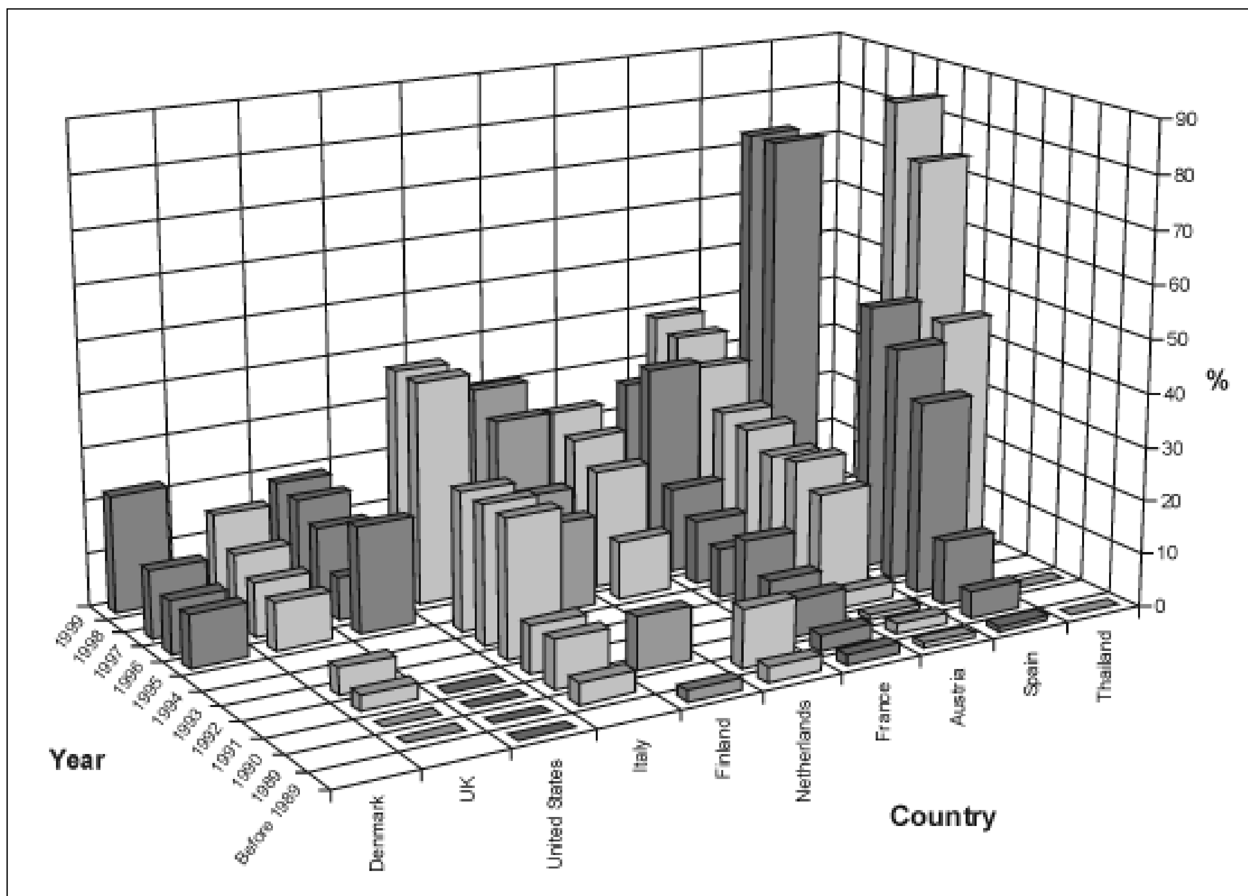


Figure 2. Trends for quinolone resistance rates (in percentages) among *Campylobacter coli* and *C. jejuni* combined from human sources around the world. The bars represent both nalidixic acid and fluoroquinolone resistance and are based on mean values of resistance from numerous reports (9,17,24,27,39,43,56-58,61-64,72-75,78,88, plus pers. comm. from Feirel G and Rautelin H, and unpub. data from Nachamkin I).

and species (Table 2). Almost all studies report a higher frequency of erythromycin resistance in *C. coli* than in *C. jejuni* (0% to 11% in *C. jejuni* vs. 0% to 68.4% in *C. coli)*. Trends over time for erythromycin resistance show stable and low rates in Japan, Canada, and Finland, but recent development of resistance in Thailand and Sweden (45,73).

Trends over Time for Quinolone Resistance

Resistance to fluoroquinolones in *Campylobacter* has clearly increased over the past decade in many parts of the world (Figure 2). Before 1989, resistance was rare. With the introduction of enrofloxacin in veterinary medicine (Table 1) and (probably less important) fluoroquinolones in human medicine in mainland Europe (the Netherlands, Finland, France, and Spain), a rapid emergence of quinolone resistance in *Campylobacter* isolates from patients was noted (8,9,43,55,64,89,90).

Surveillance data on resistance rates in human isolates from Asia soon indicated an equal increase (84,91). Quinolones were approved for veterinary use in the United Kingdom and the United States in late 1993 and 1995, respectively; reports from these areas now show increasing quinolone-resistance profiles (10,39,88).

In the latest data from Taiwan, Thailand, and Spain, rates of fluoroquinolone resistance in *C. jejuni*, or *C. jejuni* and C. *coli* combined were 56.9%, 84%, and 75% to 88%, respectively (27,31,40,73). In these regions, where quinolone resistance is highly endemic and *Campylobacter* spp. predominate, fluoroquinolones cannot be recommended for community-acquired bacterial diarrhea. Although lower frequencies are reported from other regions, recent trends show a clear tendency of emerging quinolone resistance in many countries. Quinolone resistance in human isolates often coincides with or follows the approval of fluoroquinolones for use in animal husbandry (Table 1, Figure 2), although some differences in resistance rates between countries may be explained by differences in association with foreign travel, commerce, methods of testing, and surveillance activity.

Multidrug Resistance

Multidrug resistance to macrolides and fluoroquinolones must be considered highly undesirable in *Campylobacter* as these two classes are generally advocated as first- and second-line drugs for antimicrobial treatment of *Campylobacter* enteritis.

Additional resistance to other relevant therapeutic agents poses a risk when there is no effective antimicrobial regimen for *Campylobacter* infections. Recently, Hoge et al. (73) found 100% co-resistance between Thai isolates resistant to azithromycin and ciprofloxacin in the last 2 years

Table 2. Erythromycin and azithromycin resistance rates (%) in *Campylobacter* in humans, worldwide, since 1989

Country	*C. jejuni*	*C. coli*	*C. jejuni* and *C. coli*	Reference
Austria	0.7	5.5	<1-1.4	(58 & pers. comm.[a])
Canada	0-3.3	-	-	(59,60)
Denmark	0	14.0	0-4	(24,61,62)
Finland	-	-	<1-3	(43,63, & pers. comm.[b])
France	1.1	12.2	3.5	(64)
Hungary	-	-	0	(65)
Italy	1.2-6	16-68.4	7.8-11.6	(66-69)
Japan	0.8	-	-	(66)
New Zealand	-	-	1.5	(70)
Singapore	-	-	51	(71)
Spain	0-11.0	0-35.0	3.2-7.3	(17,27,56,57,72)
Sweden	6.4[c]	11.1[c]	7.3[c]	(44)
Taiwan	10.0	50.0	18.3	(31)
Thailand	-	-	0-31.0	(73,74)
United Kingdom	1	13	1.8	(75)
United States	0-7.8	-	-	(10,76-78, & unpub. data[d])

[a]G Feierl, 2000, pers. comm.
[b]H Rautelin, 1999, pers. comm.
[c]90% of isolates were acquired abroad.
[d]I Nachamkin, 2000, unpub. data.

of surveillance. In addition, the level of tetracycline and ampicillin resistance in Thailand is so high that these agents now have no role in the treatment of *Campylobacter* or noncholera diarrhea. Li et al. (31) reported that concomitant resistance rates among nalidixic acid-resistant *C. jejuni* isolates from their patients (exclusively children) were as follows: gentamicin 2%, erythromycin 12%, clindamycin 12%, tetracyline 97%, and ciprofloxacin 66%. All of these human erythromycin-resistant *C. jejuni* isolates and 90% of the *C. coli* isolates were concomitantly resistant to clindamycin.

Consequences of Resistance for the Clinical Decision-making Process

Distinguishing infections caused by different enteric pathogens is seldom possible, so antimicrobial-drug use in the clinical setting is not confined to the treatment of *Campylobacter* spp. but rather to empiric treatment of community-acquired diarrhea in general. Increased rates of resistance have also been reported from nontyphoidal salmonellae (25,92), and documented failures in the treatment of human *Salmonella* infections have been described (93). Therefore, having continuous information on the resistance patterns of the most common bacteria causing gastrointestinal infections is critical.

Control Measures

Surveillance of resistance in *Campylobacter* is of paramount importance when fluoroquinolones are used to treat human infections. Systematic surveillance and timely reporting of antibiotic resistance patterns in *C. jejuni* and *C. coli* and other enteric pathogens from different regions should become a high priority. The principal purpose of monitoring antimicrobial resistance trends in enteric pathogens is to provide clinicians with data that can be used to select appropriate treatment regimens. Surveillance should emphasize antibiotics that are being used routinely to treat diarrhea, as well as any alternatives, such as fluoroquinolones, macrolides, and gentamicin. Equally important is the accessibility of the data to those providing primary care. For quinolones, quantitative nalidixic acid susceptibility data are more sensitive than fluoroquinolone susceptibility data for detecting common first-step mutations causing reduced susceptibility.

To circumvent the development of resistance, we have two options: infection control (zoonoses control) and prudent use of antibiotics. Improved infection control strategies along the chain “stable to the table” and guidelines for prudent use of antimicrobial agents in food animal production should be developed (94,95). To prevent further development of resistance in *Campylobacter,* limiting the use of macrolides and fluoroquinolones for food animals as much as possible is recommended. In Denmark, fluoroquinolones are not essential for treatment of any type of infection in food animals, according to surveillance performed by the Danish Veterinary Laboratory, and their use is only recommended on the rare occasion when no other therapeutic option is available (22). Because of the selection for resistance, the use of macrolides for growth promotion has been banned in all European Union countries since July 1999. The effect on the occurrence of resistance in bacteria in food animals is still not known. However, preliminary results suggest that macrolide resistance in *C. coli* from pigs in Denmark has decreased along with the decreased use of tylosin (FM Aarestrup, unpub. data).

Conclusions

Review of in vitro macrolide and quinolone resistance prevalence and trends in *Campylobacter* isolated from humans showed a temporal relationship between use of quinolones in food animals and resistant *Campylobacter* isolates in humans. The public health effects of antibiotic use in agricultural practice, including resistance of *C. jejuni* and *C. coli* to macrolides and quinolones, should be estimated. Adequate actions for control are strongly needed in both veterinary and human medicine. The public health issue of resistance in *Campylobacter* has global dimensions because of ever-increasing international trade and travel.

This work was supported in part by the Natural Sciences and Engineering Research Council of Canada to D.E.T., a medical scientist with the Alberta Heritage Foundation for Medical Research.

Dr. Engberg is a physician at the Danish national reference laboratory for enteric pathogens at Statens Serum Institut. His research interests focus on the epidemiologic, antimicrobial susceptibility, and molecular typing aspects of *Campylobacter*.

References

1. Tauxe RV. In: Nachamkin I, Blaser MJ, Tompkins LS, editors. *Campylobacter jejuni*: current status and future trends. Washington: American Society for Microbiology; 1992. p. 9-19.
2. Blaser MJ, Mandell GL, Bennett JE, Dolin R, editors. Principles and practice of infectious diseases. 4th ed. New York: Churchill Livingstone Inc.;1995. p. 1948-56.
3. Allos BM, Blaser MJ. *Campylobacter jejuni* and the expanding spectrum of related infections. Clin Infect Dis 1995;20:1092-9.
4. Dryden MS, Gabb RJ, Wright SK. Empirical treatment of severe acute community-acquired gastroenteritis with ciprofloxacin. Clin Infect Dis 1996;22:1019-25.
5. Skirrow MB, Blaser MJ, Blaser MJ, Smith PD, Ravdin JI, Greenberg HB, et al. editors. Infections of gastrointestinal tract. New York: Raven Press; 1995. p. 825-48.
6. Wistrom J, Norrby SR. Fluoroquinolones and bacterial enteritis, when and for whom? J Antimicrob Chemother 1995;36:23-39.
7. Piddock LJ. Quinolone resistance and *Campylobacter* spp. J Antimicrob Chemother 1995;36:891-8.
8. Adler-Mosca H, Lüthy-Hottenstein J, Martinetti Lucchini G, Burnens A, Altwegg M. Development of resistance to quinolones in five patients with campylobacteriosis treated with norfloxacin or ciprofloxacin. Eur J Clin Microbiol Infect Dis 1991;10:953-7.
9. Endtz HP, Ruijs GJ, van Klingeren B, Jansen WH, van der Reyden T, Mouton RP. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother 1991;27:199-208.
10. Smith KE, Besser JM, Hedberg CW, Leano FT, Bender JB, Wicklund JH, et al. Quinolone-resistant *Campylobacter jejuni* infections in Minnesota, 1992-1998. N Engl J Med 1999;340:1525-32.
11. Prescott JF, Baggot JD. Antimicrobial therapy in veterinary medicine. 2nd ed. Ames (IA): Iowa State University Press; 1993.
12. Taylor DE. In: Nachamkin I, Blaser MJ, Tompkins LS, editors. *Campylobacter jejuni* - current status and future trends. Washington: American Society for Microbiology; 1992. p. 74-86.
13. Yan W, Taylor DE. Characterization of erythromycin resistance in *Campylobacter jejuni* and *Campylobacter coli*. Antimicrob Agents Chemother 1991;35:1989-96.
14. Taylor DE, Ge Z, Purych D, Lo T, Hiratsuka K. Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. Antimicrob Agents Chemother 1997;41:2621-8.
15. Trieber CA, Taylor DE. In: Mobley HLT, Nachamkin I, McGee D, editors. Abstracts and final program of the 10th International Workshop on *Campylobacter*, *Helicobacter* and related organisms. Baltimore: University of Maryland School of Medicine; 1999; Abstract CA6. p. 3.
16. Wang Y, Huang WM, Taylor DE. Cloning and nucleotide sequence of the *Campylobacter jejuni gyrA* gene and characterization of quinolone resistance mutations. Antimicrob Agents Chemother 1993;37:457-63.
17. Ruiz J, Goni P, Marco F, Gallardo F, Mirelis B, Jimenez De Anta T, et al. Increased resistance to quinolones in *Campylobacter jejuni*: a genetic analysis of *gyr*A gene mutations in quinolone-resistant clinical isolates. Microbiol Immunol 1998;42:223-6.
18. Gibreel A, Sjögren E, Kaijser B, Wretlind B, Sköld O. Rapid emergence of high-level resistance to quinolones in *Campylobacter jejuni* associated with mutational changes in *gyrA* and *parC*. Antimicrob Agents Chemother 1998;42:3276-8.
19. Gootz TD, Martin BA. Characterization of high-level quinolone resistance in *Campylobacter jejuni*. Antimicrob Agents Chemother 1991;35:840-5.
20. Charvalos E, Tselentis Y, Hamzehpour MM, Köhler T, Pechere J-C. Evidence for an efflux pump im multidrug-resistant *Campylobacter jejuni*. Antimicrob Agents Chemother 1995;39:2019-22.
21. van Diest J, de Jong A. Overview of quinolone usage for food-producing animals. In: Use of quinolones in food animals and potential impact on human health. Report and proceedings of a WHO meeting. Geneva: World Health Organization; 1999. p.97.
22. Antibiotic resistance in the European Union associated with therapeutic use of veterinary medicines. Report and qualitative risk assessment by the committee for veterinary medical products. London: The European Agency for the Evaluation of Medical Products; 1999.
23. Jacobs Reitsma WF, Kan CA, Bolder NM. The induction of quinolone resistance in *Campylobacter* in broilers by quinolone treatment. Lett Appl Microbiol 1994;19228-31.
24. Aarestrup FM, Nielsen EM, Madsen M, Engberg J. Antimicrobial susceptibility patterns of thermophilic *Campylobacter* spp. from humans, pigs, cattle, and broilers in Denmark. Antimicrob Agents Chemother 1997;41:2244-50.
25. Bager F, editor. Danmap 98 - Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen, Denmark: Danish Zoonosis Centre; 1999. p.3.
26. Cabrita J, Rodriguez J, Braganca F, Morgado C, Pires I, Penha Goncalves A. Prevalence, biotypes, plasmid profile and antimicrobial resistance of *Campylobacter* isolates from wild and domestic animals from Northeast Portugal. J Appl Microbiol 1992;73:279-85.
27. Saenz Y, Zarazaga M, Lantero M, Gastanares MJ, Baquero F, Torres C. Antibiotic resistance in *Campylobacter* strains isolated from animals, foods, and humans in Spain in 1997-1998. Antimicrob Agents Chemother 2000;44:267-71.
28. Moore JE, Madden RH, Kerr JR, Wilson TS, Murphy PG. Erythromycin-resistant thermophilic *Campylobacter* species isolated from pigs [see comments]. Vet Rec 1996;138:306-7.
29. Nielsen EM, Nielsen NL. Serotypes and typability of *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry products. Int J Food Microbiol 1999;46:199-205.
30. Endtz HP, Mouton RP, van der Reyden T, Ruijs GJ, Biever M, van Klingeren B. Fluoroquinolone resistance in *Campylobacter* spp. isolated from human stools and poultry products [letter] [see comments]. Lancet 1990;335:787.

31. Li CC, Chiu CH, Wu JL, Huang YC, Lin TY. Antimicrobial susceptibilities of *Campylobacter jejuni* and *coli* by using E-test in Taiwan. Scand J Infect Dis 1998;30:39-42.
32. Gaunt PN, Piddock LJ. Ciprofloxacin resistant *Campylobacter* spp. in humans: an epidemiological and laboratory study. J Antimicrob Chemother 1996;37:47-57.
33. Skirrow MB, Blaser MJ. In: Nachamkin I, Blaser MJ, Tompkins LS, editors. *Campylobacter jejuni*: current status and future trends. Washington: American Society for Microbiology; 1992. p. 3-8.
34. Neimann J, Engberg J, Mølbak K, Wegener HC. 4th World Congress on Foodborne Infections and Intoxications (Proceedings). Berlin: Federal Institute for Health Protection of Consumer and Veterinary Medicine; 1998. p. 298-303.
35. Engberg J, Gerner-Smidt P, Scheutz F, Nielsen EM, On SLW, Mølbak K. Water-borne *Campylobacter jejuni* infection in a Danish town—a 6-week continuous source outbreak. Clin Microbiol Infect 1998;4:648-56.
36. On SLW, Nielsen EM, Engberg J, Madsen M. Validity of *Sma*I-defined genotypes of *Campylobacter jejuni* examined by *Sal*I, *Kpn*I, and *Bam*HI polymorphisms: evidence of identical clones infecting humans, poultry, and cattle. Epidemiol Infect 1998;120:231-7.
37. Nielsen EM, Engberg J, Madsen M. Distribution of serotypes of *Campylobacter jejuni* and *C. coli* from Danish patients, poultry, cattle and swine. FEMS Immunol Med Microbiol 1997;19:47-56.
38. Doyle MP, Jones DM. In: Nachamkin I, Blaser MJ, Tompkins LS, editors. *Campylobacter jejuni* - current status and future trends. Washington: American Society for Microbiology; 1992. p. 45-8.
39. Piddock LJV. Working Paper 20.09. Geneva: World Health Organization; 1998. p. 1-9.
40. Gallardo F, Gascon J, Ruiz J, Corachan M, de Anta MTJ, Vila J. *Campylobacter jejuni* as a cause of traveler's diarrhea: clinical features and antimicrobial susceptibility. J Travel Med 1998;5:23-6.
41. Mattila L, Peltola H, Siitonen A, Kyronseppa H, Simula I, Kataja M. Short-term treatment of traveler's diarrhea with norfloxacin: a double-blind, placebo-controlled study during two seasons. Clin Infect Dis 1993;17:779-82.
42. Friedman CR, Yang S, Rocourt J, Stamey K, Vugia D, Marcus R, et al. In: 36th annual meeting of the Infectious Diseases Society of America - Program and Abstracts. Denver, Colorado: The Infectious Diseases Society of America; 1998; Abstract 545 Fr, p. 179.
43. Rautelin H, Renkonen OV, Kosunen TU. Emergence of fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli* in subjects from Finland. Antimicrob Agents Chemother 1991;35:2065-9.
44. Sjögren E, Kaijser B, Werner M. Antimicrobial susceptibilities of *Campylobacter jejuni* and Campylo*bacter coli* isolated in Sweden: a 10-year follow-up report. Antimicrob Agents Chemother 1992;36:2847-9.
45. Sjögren E, Lindblom GB, Kaijser B. Norfloxacin resistance in *Campylobacter jejuni* and *Campylobacter coli* isolates from Swedish patients. J Antimicrob Chemother 1997;40:257-61.
46. Engberg J, Andersen S, Skov R, Aarestrup FM, Gerner-Smidt P. Comparison of two agar dilution methods and three agar diffusion methods including the E-test for antibiotic susceptibility testing of thermophilic *Campylobacter* species. Clin Microbiol Infect 1999;5:580-4.
47. Orr KE, Lightfoot NF, Sisson PR, Harkis BA, Tveddle JL, Boyd P, et al. Direct milk excretion of *Campylobacter jejuni* in a dairy cow causing cases of human enteritis. Epidemiol Infect 1995;114:15-24.
48. Pearson AD, Greenwood MH, Donaldson J, Healing TD, Jones DM, Shahamat M, et al. Continuous source outbreak of campylobacteriosis traced to chicken. J Food Prot 2000;63:309-14.
49. Owen RJ, Leeton S. Restriction fragment length polymorphism analysis of the flaA gene of *Campylobacter jejuni* for subtyping human, animal and poultry isolates. FEMS Microbiol Lett 1999;176:345-50.
50. Funke G, Baumann R, Penner JL, Altwegg M. Development of resistance to macrolide antibiotics in an AIDS patient treated with clarithromycin for *Campylobacter jejuni* diarrhea. Eur J Clin Microbiol Infect Dis 1994;13:612-5.
51. Segreti J, Gootz TD, Goodman LJ, Parkhurst GW, Quinn JP, Martin BA, et al. High-level quinolone resistance in clinical isolates of *Campylobacter jejuni*. J Infect Dis 1992;165:667-70.
52. Tee W, Mijch A. *Campylobacter jejuni* bacteremia in human immunodeficiency virus (HIV)-infected and non-HIV-infected patients: comparison of clinical features and review. Clin Infect Dis 1998;26:91-6.
53. Ellis-Pegler RB, Hyman LK, Ingram RJ, McCarthy M. A placebo controlled evaluation of lomefloxacin in the treatment of bacterial diarrhoea in the community. J Antimicrob Chemother 1995;36:259-63.
54. Molina J, Casin I, Hausfater P, Giretti E, Welker Y, Decazes J, et al. *Campylobacter* infections in HIV-infected patients: clinical and bacteriological features. AIDS 1995;9:881-5.
55. Chatzipanagiotou S, Papavasiliou E, Malamou Lada E. Isolation of *Campylobacter jejuni* strains resistant to nalidixic acid and fluoroquinolones from children with diarrhea in Athens, Greece [letter]. Eur J Clin Microbiol Infect Dis 1993;12:566-8.
56. Reina J, Ros MJ, Serra A. Susceptibilities to 10 antimicrobial agents of 1,220 *Campylobacter* strains isolated from 1987 to 1993 from feces of pediatric patients. Antimicrob Agents Chemother 1994;38:2917-20.
57. Sanchez R, Fernandez Baca V, Diaz MD, Munoz P, Rodriguez Creixems M, Bouza E. Evolution of susceptibilities of *Campylobacter* spp. to quinolones and macrolides. Antimicrob Agents Chemother 1994;38:1879-82.
58. Feierl G, Berghold C, Furpass T, Marth E. Further increase in ciprofloxacin-resistant *Campylobacter jejuni/coli* in Styria, Austria. Clin Microbiol Infect 1999;5:59-60.
59. Gaudreau C, Gilbert H. Antimicrobial resistance of clinical strains of *Campylobacter jejuni* subsp. *jejuni* isolated from 1985 to 1997 in Quebec, Canada. Antimicrob Agents Chemother 1998;42:2106-8.

60. Gaudreau C, Gilbert H. In: Mobley HLT, Nachamkin I, McGee D, editors. Abstracts and final program of the 10th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Baltimore: University of Maryland School of Medicine; 1999. Abstract CA3, p. 2.
61. Bager F, editor. DANMAP 97 - Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen: Danish Zoonosis Centre; 1998. p.3.
62. Bager F, editor. DANMAP 99 - Consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen: Danish Zoonosis Centre; 2000.
63. Hänninen ML, Pajarre S, Klossner ML, Rautelin H. Typing of human *Campylobacter jejuni* isolates in Finland by pulsed-field gel electrophoresis. J Clin Microbiol 1998;36:1787-9.
64. Megraud F. Les infections à *Campylobacter* en France (1986-1997). Bulletin Epidémiologique Annuel 1998;2:83-4.
65. Varga J, Fodor L. Biochemical characteristics, serogroup distribution, antibiotic susceptibility and age-related significance of *Campylobacter* strains causing diarrhoea in humans in Hungary. Zentralbl Bakteriol 1998;288:67-73.
66. Itoh T, Tadano K, Obata H, Shingaki K, Kai A, Saito K, et al. Emergence of quinolone-resistance in clinical isolates of *Campylobacter jejuni* in Japan. In: Newell DG, Ketley J, Feldman RA, editors. 8th International Workshop on Campylobacters, Helicobacters and Related Organisms. Abstracts from the meeting held at Winchester, United Kingdom, 10th-13th July 1995. New Haw, Addlestone, England: Central Veterinary Laboratory; 1995. p. 83.
67. Piersimoni C, Crotti D, Nista D, Bornigia G, de Sio G. In: Newell DG, et al., editors. 8th International Workshop on Campylobacters, Helicobacters and Related Organisms. Abstracts from the meeting held at Winchester, United Kingdom, 10th - 13th July 1995. New Haw, Addlestone, England: Central Veterinary Laboratory; 1995; p. 88.
68. Crotti D, Medori MC, Fonzo G, Del Sante M, Silvestrini R. Clinical microbiology of Campylobacter enteritis in our experience. Clin Microbiol Infect 1999;7(Suppl. 3):267.
69. Crotti D, Fonzo G, D'Annibale ML, Medori, MC Luzzi I, Mobley HLT, et al., In: Mobley HLT, Nachamkin I, McGee D, editors. Abstracts and final program of the 10th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Baltimore: University of Maryland School of Medicine; 1999. Abstract CA7. p. 4.
70. Dowling J, MacCulloch D, Morris AJ. Antimicrobial susceptibility of *Campylobacter* and *Yersinia enterocolitica* isolates [letter]. N Z Med J 1998;111:281.
71. Lim YS, Tay L. A one-year study of enteric *Campylobacter* infections in Singapore. J Trop Med Hyg 1992;95:119-23.
72. Navarro F, Miro E, Mirelis B, Prats G. *Campylobacter* spp. antibiotic susceptibility [letter; comment]. J Antimicrob Chemother 1993;32:906-7.
73. Hoge CW, Gambel JM, Srijan A, Pitarangsi C, Echeverria P. Trends in antibiotic resistance among diarrheal pathogens isolated in Thailand over 15 years. Clin Infect Dis 1998;26:341-5.
74. Murphy GS, Jr, Echeverria P, Jackson LR, Arness MK, LeBron C, Pitarangsi C. Ciprofloxacin- and azithromycin-resistant *Campylobacter* causing traveler's diarrhea in U.S. troops deployed to Thailand in 1994. Clin Infect Dis 1996;22:868-9.
75. Frost JA, Thwaites RT. Drug resistance in *C. jejuni, C. coli* and *C. lari* isolated from humans in Wales and North West England during 1997. Working Paper 20.10b. Geneva: World Health Organization; 1998.
76. Baker CN. The E-Test and *Campylobacter jejuni*. Diagn Microbiol Infect Dis 1992;15:469-72.
77. National antimicrobial resistance monitoring system NARMS - 1997 annual report revised. Atlanta: Centers for Disease Control and Prevention; 1998.
78. Nachamkin I. Antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli* to ciprofloxacin, erythromycin and tetracycline from 1982 to 1992. Med Microbiol Lett 1992;2:300-5.
79. Hirschl AM, Wolf D, Berger J, Rotter ML. In vitro susceptibility of *Campylobacter jejuni* and *Campylobacter coli* isolated in Austria to erythromycin and ciprofloxacin. Zentralbl Bakteriol 1990;272:443-7.
80. Feierl G, Pschaid A, Sixl B, Marth E. Increase of ciprofloxacin resistance in *Campylobacter* species in Styria, Austria. Int J Med Microbiol Virol Parasitol Infect Dis 1994;281:471-4.
81. Stobberingh E, van den Bogaard A, Mevius D, Endtz H. Examples of *in-vitro* quinolone resistance prevalence trends in humans and animal isolates of food-borne *Salmonella* and *Campylobacter*. Working Paper 20.09. Geneva: World Health Organization; 1998.
82. Perez Trallero E, Urbieta M, Lopategui CL, Zigorraga C, Ayestaran I. Antibiotics in veterinary medicine and public health [letter; comment]. Lancet 1993;342:1371-2.
83. Reina J. Resistance to fluoroquinolones in *Salmonella* non-typhi and *Campylobacter* spp. [letter; comment]. Lancet 1992;340:1035-6.
84. Kuschner RA, Trofa AF, Thomas RJ, Hoge CW, Pitarangsi C, Amato S, et al. Use of azithromycin for the treatment of *Campylobacter* enteritis in travelers to Thailand, an area where ciprofloxacin resistance is prevalent. Clin Infect Dis 1995;21:536-41.
85. Bowler I, Day D. Emerging quinolone resistance in campylobacters [letter; comment]. Lancet 1992;340:245.
86. McIntyre M, Lyons M. Resistance to ciprofloxacin in *Campylobacter* spp. [letter; comment]. Lancet 1993;341:188.
87. Bowler IC, Connor M, Lessing MP, Day D. Quinolone resistance and *Campylobacter* species [letter]. J Antimicrob Chemother 1996;38:315.
88. Sam WIC, Lyons MM, Waghorn DJ. Increasing rates of ciprofloxacin resistant *Campylobacter* [letter]. J Clin Pathol 1999;52:709.

89. Shah PM, Schafer V, Knothe H. Medical and veterinary use of antimicrobial agents: implications for public health. A clinician's view on antimicrobial resistance. Vet Microbiol 1993;35:269-74.
90. Reina J, Borrell N, Serra A. Emergence of resistance to erythromycin and fluoroquinolones in thermotolerant *Campylobacter* strains isolated from feces 1987-1991. Eur J Clin Microbiol Infect Dis 1992;11:1163-6.
91. Tee W, Mijch A, Wright E, Yung A. Emergence of multidrug resistance in *Campylobacter jejuni* isolates from three patients infected with human immunodeficiency virus. Clin Infect Dis 1995;21:634-8.
92. Threlfall EJ, Ward LR, Rowe B. Resistance to ciprofloxacin in non-typhoidal salmonellas from humans in England and Wales - the current situation. Clin Microbiol Infect 1999;5:130-4.
93. Mølbak K, Baggesen DL, Aarestrup FM, Ebbesen JM, Engberg J, Frydendahl K, et al. An outbreak of multidrug-resistant, quinolone-resistant *Salmonella* enterica serotype Typhimurium DT 104. N Engl J Med 1999;341:1420-5.
94. Pedersen KB, Aarestrup FM, Jensen NE, Bager F, Jensen LB, Jorsal SE, et al. The need for a veterinary antibiotic policy. Vet Rec 1999;(July 10):50-3.
95. Use of quinolones in food animals and potential impact on human health. Report of a WHO meeting, Geneva, Switzerland, 2-5 June 1998. Geneva: World Health Organization; 1998.

Geographic Subdivision of the Range of the Malaria Parasite *Plasmodium vivax*

Jun Li,* William E. Collins,† Robert A. Wirtz,† Dharmendar Rathore,* Altaf Lal,† and Thomas F. McCutchan*
*National Institutes of Health, Bethesda, Maryland, USA; and
†Centers for Disease Control and Prevention, Atlanta, Georgia, USA

We examined geographically distinct isolates of *Plasmodium vivax* and categorized them according to developmental success in *Anopheles albimanus*. We found that parasites from Central America and Colombia form a group distinct from those of Asia. New World isolates have a distinct chromosomal translocation and an episomal variation in the open reading frame (ORF) 470 DNA sequence that distinguishes them from the other isolates tested. Old World types of *P. vivax* were introduced into the Americas, and a remnant of this lineage remains in *P. simium*. It is indistinguishable from Old World *P. vivax* to the extent determinable by using our encoded markers and the examination of its developmental pattern in mosquitoes. The cohesive characteristics that separate types of *P. vivax* are predictors of range and potential for transmission and hence require taxonomic distinction.

Plasmodium vivax is the most common of four human malaria species, with a worldwide distribution within approximately 16° to 20° north and south of the summer isotherms. Before its unexplained disappearance from Europe, *P. vivax* was probably present as far north as Moscow. Currently the organism is endemic in many countries of Asia, the South Pacific, North Africa, the Middle East, and South and Central America (1).

The biologic diversity included within this species designation has justified the use of a trinomial system for naming, including the subspecies, a taxonomic character given formal recognition in the International Rules of Zoological Nomenclature. The concepts of "species" and "subspecies" are still hotly debated, camps often dividing between those referred to as lumpers and splitters. Although species has various definitions, most depend on whether populations share or do not share a common gene pool. A subspecies is a population or group of populations inhabiting a geographic subdivision of the range of a species and differing from other populations by diagnostic morphologic characteristics. It follows that subspecies cannot be sympatric, as interbreeding would lead to the loss of identity (2). Some researchers think that distribution can be either geographically or ecologically based (3).

Address for correspondence: Thomas F. McCutchan, NIAID, National Institutes of Health, 4 Center Drive, Room 4/126, Bethesda, Maryland 20892, USA; fax: 301-402-0079; e-mail: tmccutchan@niaid.nih.gov.

In this study, we describe a large number of New and Old World *P. vivax* isolates. To assess a measure of relationship between 10 different parasite isolates, we measured the parasite infectivity of >25,000 mosquitoes in a 10-year period, using a ratio of infectivity of *Anopheles albimanus* to that of an internal control as a measure of success. We also identified and monitored a genomic and an organellar polymorphic marker and applied these to 17 different isolates from a wide geographic range. Our results were totally consistent with the geographic separation based on developmental differences in the mosquito. We determined that independently isolated strains of New World *P. vivax* are very similar to each other and, as a group, are distinct from a broad distribution of isolates collected in Asia and Oceania. This is the first report of genomic, organellar, and phenotypic markers coalescing along geographic boundaries and justifies the naming of a new subspecies of *P. vivax*.

Geographic Differences in Development of *P. vivax* Isolates

We examined the transmissibility of different isolates of *P. vivax* and found that they vary in a selective way. The comparative feeding results from the study of 10 individual strains of *P. vivax* are shown (Figure 1). For all parasite strains collected from the New World infected mosquitoes (as measured by oocyst count), the average infection rate was 30.6% for a single line of Central American *An. albimanus* and 51.9% for a laboratory line of *An. freeborni*, the positive control. The Old World parasite strains, on the other hand, had an average infection rate of only 0.25% for *An. albimanus*, while infecting the positive control, *An. freeborni,* quite normally at 63.4%. Methods and preliminary data on the correlation between different mosquito colonies and parasite development in *An. culicifacies* have been reported (5).

Further experiments tested the effect of the origin of *An. albimanus* on transmissibility of *P. vivax.* Five New World *An. albimanus* colonies were far more susceptible to each New World parasite than to any Old World parasite line (Table). The average infection rate for five different New World colonies was 21.2%. As the positive control, *An. freeborni* was infected by parasites from the different areas with a mean infection rate of 57.1%. Hence, the distinction of developmental success in *An. albimanus* did not relate to characteristics of a single colony but was a more general phenomenon. The single exception to the Old World-New World separation was *P. simium,* a New World monkey parasite morphologically similar to *P. vivax,* which has been reported to successfully infect *An. freeborni* but not to be very infective to *An. albimanus* (6).

Separation of New and Old World *P. vivax* Malaria Indicated by Analysis of Nuclear-Encoded and Plastid DNA Markers

P. vivax isolates representing different geographic areas and one isolate of *P. simium*, a parasite of New World monkeys, were

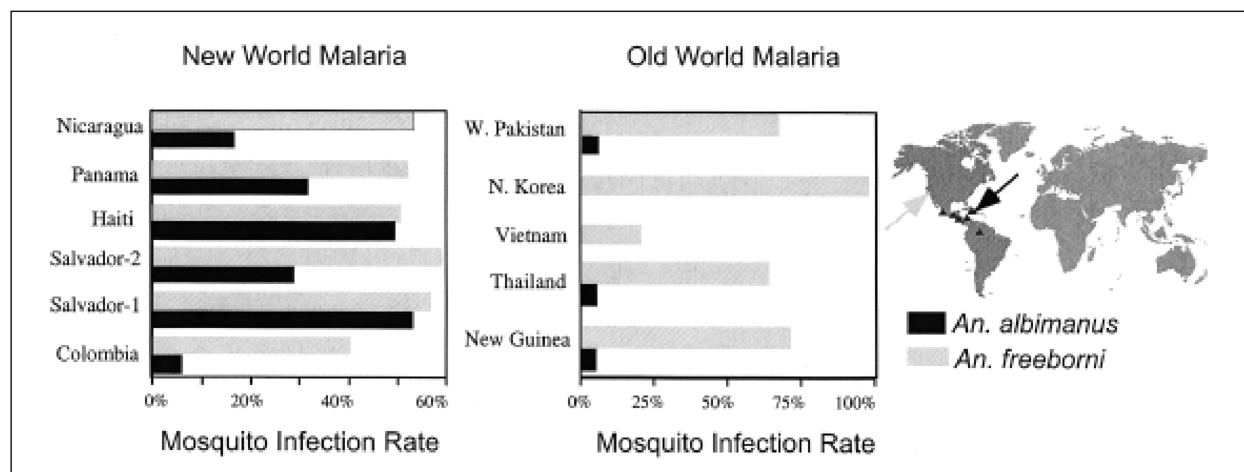


Figure 1. Comparison of the relative developmental success of 11 different isolates of *Plasmodium vivax* in *Anopheles albimanus* and *An. freeborni*. The black bars and arrow represent *An. albimanus,* and the gray bars and arrow represent *An. freeborni.* The origin of each isolate is indicated on the map. American Type Culture collection reference numbers are as follows: N. Korea T1206, Thai K1294, Vietnam C30151, New Guinea C30060, Nicaragua 30073, Panama C30138, and El Salvador-1 C30197. W. Pakistan, Salvador-2, and Colombia came directly from the Centers for Disease Control and Prevention (CDC). Analysis of feeding and infectivity are as described (4).*

*Susceptibility of *An. albimanus* to different strains of *P. vivax* was tested by feeding the mosquito on infected *Aotus* monkeys (4). A total of six strains of *An. albimanus* collected from Panama, El Salvador, Colombia, and Haiti were used to test each of 11 strains of *P. vivax*, 6 strains of which were from the New World, including 2 isolates from El Salvador and 1 each from Colombia, Haiti, Panama, and Nicaragua; 5 strains were from the Old World, including Chesson strain (New Guinea), West Pakistan strain (Pakistan), North Korea strain (Korea), Pakchong strain (Thailand), and Vietnam II strain (Vietnam). The infection rate was determined by dissecting and counting midgut oocysts or salivary glands during the second week after the infectious blood meal. *An. freeborni*, which were originally from Marysville, California, and have been maintained in the laboratory at CDC since 1944, were used as a positive control because this laboratory-selected colony has a high level of susceptibility to all strains of *P. vivax*.

Table. *Anopheles albimanus* exhibits greater susceptibility to New World versus Old World *Plasmodium vivax*

P. vivax	Mosquito	Infection rate (No. positive/no. fed)
New World	*An. albimanus*	21.2% (5,888/27,700)
	An. freeborni	57.1% (13,227/25,555)
Old World	*An. albimanus*	0.4% (10/2,508)
	An. freeborni	57% (1,790/3,135)

characterized according to sequence polymorphisms within the nuclear-encoded rRNA genes and the 35-kb plastid genome. The New World *P. vivax* isolates were identical under these criteria, while the Old World *P. vivax* and *P. simium* also formed a distinct and related group. These parasites include most of the original parasite lines tested for developmental success in *An. albimanus.*

Consistent Differences Between Old and New World Isolates of *P. vivax* in S-type rRNA Genes: Result of a Single-Type Gene Conversion

DNA sequence analysis of the cloned 18S rRNA genes from 17 isolates, including 5 of 7 Old World isolates and 5 of 7 New World isolates from infectivity studies, indicated that all strains shared similar sequence types, including 3 types of genomic rRNA genes as we described for the Sal-1 strain (7,8). In comparing the 18S rRNA sequences of New World and Old World isolates, however, we found a consistent polymorphism that separated the two according to geographic location (Figure 2). While the genes expressed in blood stage (A type) and oocysts (O type) remained the same in all isolates, the S-type genes transcribed in sporozoites were maintained in either one or the other of two distinct forms (Figure 2). The New World type of S gene has been reported in a *P. vivax* isolate from El Salvador (12), and the Old World type has been reported to exist in the monkey malaria, *P. simium* (13; Li and McCutchan, unpub. data). The difference between the two types of S gene is localized at the 3' end of the gene, including the last variable region, V8 (9). The A gene and two types of the S genes were differentiated on the basis of fragment length (Figure 2). The assignment of type was confirmed in all isolates by DNA sequence analysis. The results indicate that one type of S gene is shared by all strains or isolates from Central and South America. Parasites from Asia, the Pacific region, and Africa have a genotype similar to the S gene from the primate malaria *P. cynomolgi* and exactly like that of *P. simium.*

The structure of the variant New World S gene appeared to be the result of a simple conversion between the A and S genes (Figure 3). Further, the point of conversion between A and S genes appeared to be the same (within 9 bp) in all New World isolates. The simplest conclusion is that only one conversion occurred, and it existed in the progenitor of all New World strains thus far examined. Expression of the two types of S gene has been confirmed by sequencing of cloned reverse transcriptase/polymerase chain reaction fragments amplified from sporozoite rRNA (Li and McCutchan, data not shown).

Analysis of the Open Reading Frame (ORF) 470 in *P. vivax* Consistent with Parasite Associations Established with Genomic Markers

This 35-kb circular DNA is maternally inherited and highly conserved in sequence (14). We investigated the ORF 470, the small subunit rRNA, and the *Clp* gene for phylogenetically informative sites. The results show that a conserved substitution of the ORF 470 has been maintained among the New World *P. vivax*, where an isoleucine is replaced by valine. The parasites from Old World isolates maintain the isoleucine, as do their putative progenitors from primate malarias (Figure 4). The limited number of maternal types is not unexpected because of the extensive plastid homogeneity found within species of these parasites (14). We assume that this polymorphism is representative of two separate lineages.

Discussion

The evolution of a new species or subspecies from within the range of a single sexually reproducing population has been of central interest to biologists since before Darwin. With regard to medicine and epidemiology, an understanding of the above would substantially contribute to designing procedures for pathogen control (15). We have shown that a partition subdivides the population range of *P. vivax* and have presented one reason why such partitions remain stably in place.

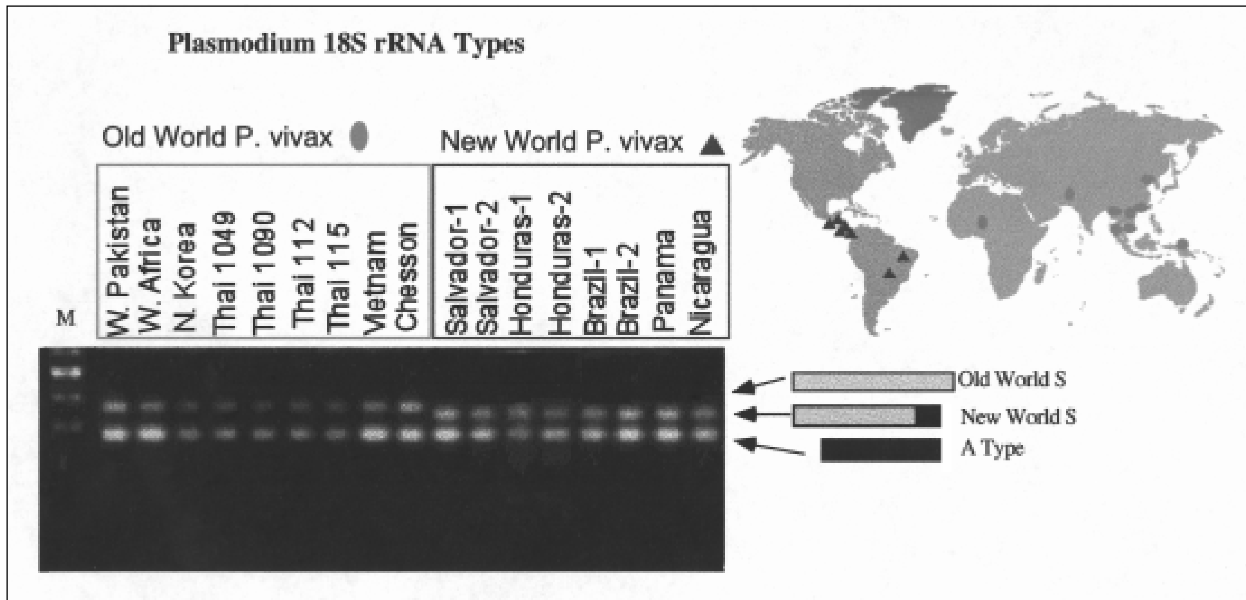


Figure 2. Sequences of *Plasmodium vivax* isolates are distinguished by variation in the 3' end of the S-type rRNA gene (9). The S-type gene is longer in Old World isolates and in *P. simium*. Oligonucleotide #902 (5'CAG CAAGCTGAATCGTAATTTTAA3') was used to detect type A rRNA, and #743 (5'ATCCAGATCCAATCCGACATA3') and #901 (5'GATAAGCACAAAATAGCGAAATGC3') were used to differentiate the two S-type rRNAs in membrane blot hybridization. American Type Culture Collection reference numbers not designated in the Figure 1 legend are as follows: Thai R112, Thai R115, Honduras-1 T09794, Honduras-2 T10595, Brazil-1 T40695, and Thai K1090. Haiti, Brazil-2, and the West African isolates came directly from the Centers for Disease Control and Prevention (CDC).*†

*The *P. vivax* SAL-1 strain, Africa strain, Pakistan strain, Brazil strain, and New World monkey vivax, *P. simium,* were from CDC. *P. vivax* Vietnam strain, Chesson strain, and Panama strain were from the American Type Culture Collection. The four Thai strains were from Walter Reed Medical Center; they were collected from four geographically separate locations in Thailand and their immunologic characteristics described with regard to the circumsporozoite protein gene (10). The heparinized blood samples were stored in 5% glycerolyte at -70°C.

†Purified DNA from frozen blood samples was processed with DNAzol reagents (GIBCO-Bethesda Research Laboratory, Gaithersburg, MD) according to the manufacturer's instructions. The partial sequence of 18S rRNA genes that covers variable regions 7 and 8 was amplified with a pair of genus-conserved primers, #841 and #844 (9). The sequence of an open reading frame (ORF 470) on the 35-kb plastid-like DNA was amplified with a pair of oligonucleotide primers #1274 (5' GTAAAAT TATATAAACCACC 3') and #1273 (5' GCACAATTTGAACGTAC 3') conserved in plastid-like organelle in *Apicomplexa* (11). The sequence of circumsporozoite protein genes was amplified with oligonucleotide primers #1157 (5' AATGGAGTAAACT TCAATAATGTA 3') and #1160 (5' CTCCACAGGTTACACTGCATG 3'). The reaction was set up in a 100-µL reaction volume containing 20–50 ng DNA, 200 M of each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 2 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) at a three-step cycling with the following parameters: 94°C/1 min for denaturation, 50°C/1 min for annealing, and 72°C/1–2 min for elongation for a total of 30 cycles. Conditions for separation of polymerase chain reaction products on agarose electrophoresis and ^{32}P-ending labeled probe hybridization were described (8,9). Oligonucleotide #902 (5' CAGCAAGCTGAATCGTAATTTTAA 3') was used to detect type-A rRNA, and #743 (5' ATCCAGATCCAATCCGACATA 3') and #901 (5' GATAAGCACAAAATAGCGAAATGC 3') were used to differentiate the two S-type rRNAs in membrane blot hybridization. Oligonucleotide #1163 (5' AGAGCAGCTGGACAGCCAGCA 3') and #1165 (5'GCWGGCAATCAACCAGGAGCA3') were used as probes to differentiate Sal-1 (classic) and PVK247 (variant) types of circumsporozoite protein genes, respectively. The gene coding for 18S ribosomal RNA was amplified from the DNA of Thai isolates with oligonucleotides #566, 5' GGATAACTACGGAAAAGCTGTAGC 3', and #570, 5' CGACTTCTCCTTCCTTTAAAA GATAGG 3', as the 5' and 3' end primers, respectively. Both primers are conserved for the genus *Plasmodium* and cover most of the transcribed sequences from approximately 140 downstream of the 5' end to 40 bp upstream of the 3' end.

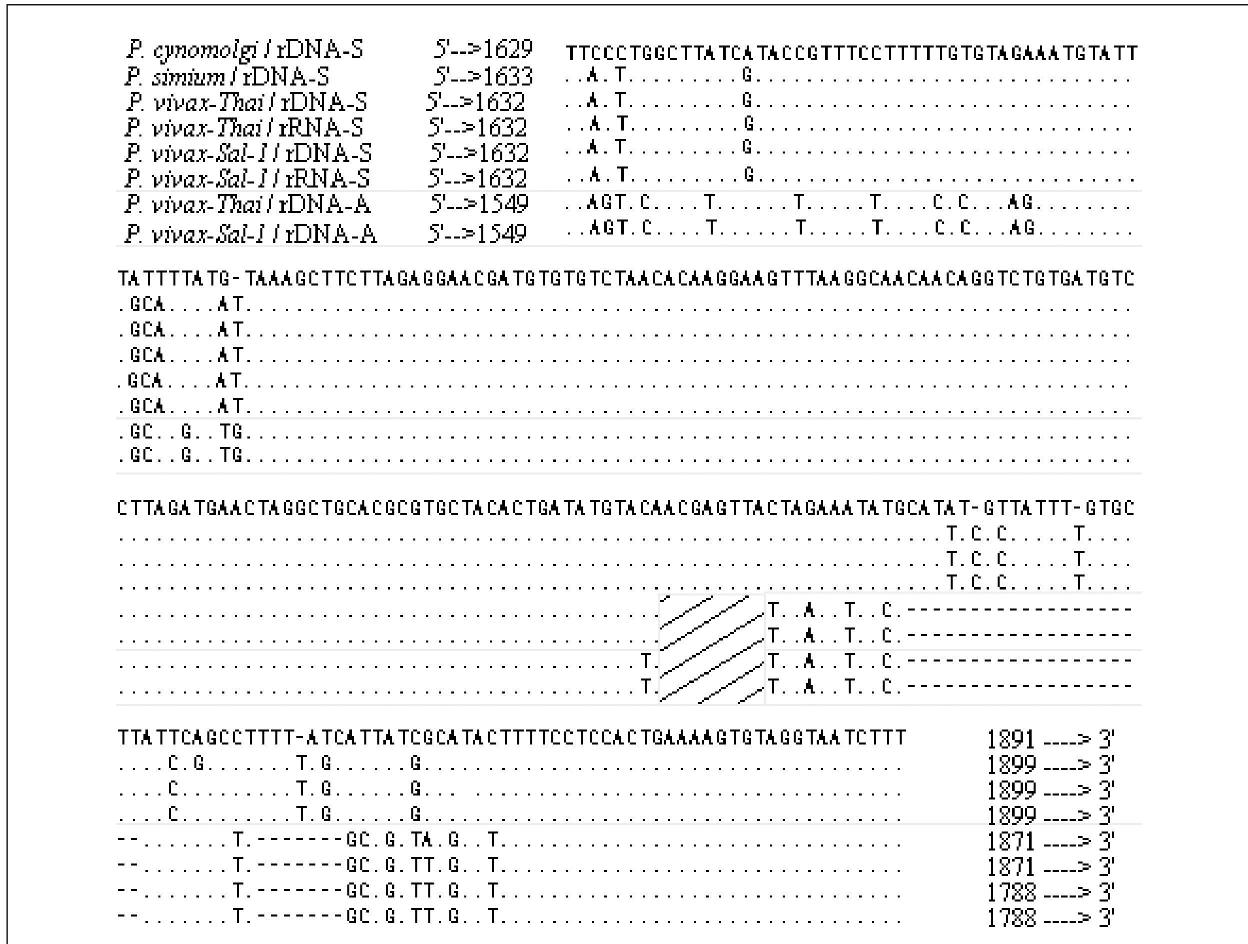


Figure 3. The sequence of *Plasmodium vivax* from the Americas is distinguished from Old World isolates by analysis of the 3' end of the S-type rRNA gene. The S-type rRNA sequences were determined from cloned amplified products of parasite DNA and RNA.

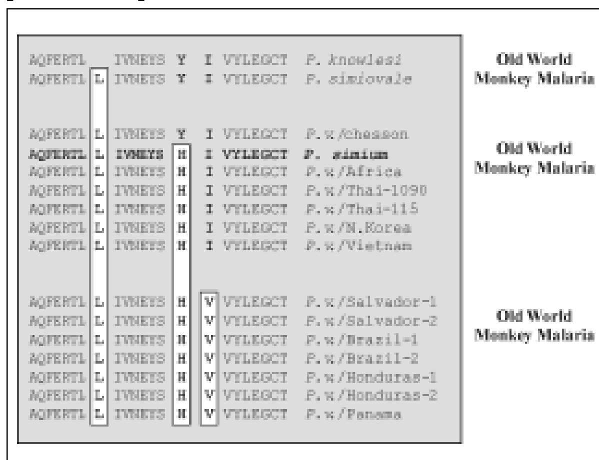


Figure 4. Polymorphism in the ORF 470 region of the 35-kb plastid-like DNA was determined by DNA sequence analysis after amplification of DNA from each isolate with oligonucleotide primers #1274 (5'GT AAAATTATATAAACCACC3') and #1273 (5'GCACA ATTTGAACGTAC3') (11).

The epidemiologic impact of barriers to genetic exchange is substantial. Such barriers can come from both geologic and biologic sources. We have shown that differing vector-parasite compatibility can create genetic isolation between populations of a single parasite species. In principle, the question of differing vector-parasite compatibility has been addressed by the observation of historic events. When millions of potentially exposed soldiers returned home from World War II, Korea, and Vietnam, reintroduction of malaria was considered a serious possibility, given that *P. vivax* was only eliminated from the United States in the 1940s and the vectors were still in abundance. The fact that *P. vivax* was not reintroduced was, at the time, surprising. How could the parasites from Asia and America be different? Because *P. vivax* collected from the New World, Africa, and Asia have indistinguishable morphologic features and

life cycles, as well as many of the same surface antigens, it was assumed that they represented the same parasite.

A dichotomy is revealed when one compares the relative developmental success of different parasite isolates in *An. albimanus* and *An. freeborni.* Both mosquitoes come from the Americas, but *An. albimanus* is indigenous to malarious areas and *An. freeborni* is not. When different isolates of *P. vivax* were fed to the New World mosquitoes, an obvious division formed along broad geographic lines. *An. albimanus* were more easily infected by New World parasites than by Old World parasites, whereas no difference in infectivity was detected in the control, *An. freeborni.* Hence, adaptation to the mosquito, rather than general developmental fitness, is the selective basis for separation. Examination of *An. albimanus* from five different areas of the New World each revealed the dichotomy. It is of interest that the insect-parasite association developed along broad geographic lines.

More than 25,000 mosquitoes were dissected during a 10-year period, and the resultant large numbers have implications for the interpretation of results. Clearly, the feedings were repeated many times, and each can be considered a separate experiment. The same protocol was followed in each experiment, but slight variations in factors such as the exact age of the mosquitoes and the source of infective blood must have occurred during the study period. The simple conclusion that a large difference exists in the developmental success of parasites from the Old and New Worlds in *An. albimanus* was entirely consistent over the period, and all experiments are in accord with that conclusion. Development of the same parasite strains was also tested in an Old World mosquito, *An. culicifacies* (Collins et al., unpub. data). As a group, Old World parasites infected *An. culicifacies* more efficiently and had a higher rate of oocyst production than parasites from the New World, although some New World parasite lines had not lost the potential to infect *An. culicifacies*. This indicates that while the lineage giving rise to New World parasites may have developed successfully in *An. culicifacies* and other Old World mosquitoes, that potential may be lost during the process of adapting to a new environment.

The developmental mechanism involved in determining success of a parasite isolate in mosquito colonies represents an unknown number of loci. Data supporting the division defined by infectivity studies were based on DNA polymorphisms in samples supplied from the American Type Culture Collection (Rockville, MD) and the Centers for Disease Control and Prevention's Division of Parasitic Diseases. The episomal marker is located within the ORF 470 of the 35-kb plastid (11), which is physically unlinked to genomic markers. Our genomic marker is a polymorphism resulting from a defined ribosomal RNA gene transformation. Although we cannot definitively say that the division of groups is related to their genetic isolation (i.e., we cannot show that we have saturated the genome with markers), that does appear to be the case. The premise of our interpretation is that episomal and chromosomal ribosomal markers are not linked to each other and are unlikely to be linked to genes controlling parasite development in the mosquito.

P. simium carries genomic and maternal polymorphisms identical to the Old World human parasite *P. vivax*. Its ancestors are certainly more maternally related to the Old World *P. vivax* of humans than to the *P. vivax* that now dominates in humans in South America. Thus, a lateral transfer occurred during *P. simium* evolution, as previously indicated (13,16,17). *P. simium* and New World *P. vivax* did not originate directly from the same source, contrary to published speculation on the parasite's evolution. Therefore, *P. vivax* most likely entered the New World on two separate occasions and from different geographic locations.

Two types of repetitive epitope have been described in the circumsporozoite protein gene of *P. vivax*; their distribution appears to be worldwide (18). We confirm this finding in our isolates (data not shown); hence, all our isolates arose from a common ancestor. The separation of these two *P. vivax* subspecies may have started before entry into the Americas, but it is likely that isolation and adaptation to the new environment were strong forces in driving the fixation of the parasites that now exist there. It is widely, although not universally, agreed that *P. vivax* entered into the Americas within the last 500 years. If true, this would suggest that fixation and dispersal over a wide geographic area happened within a few hundred years. Although this seems rapid, it is within the realm of what we know about malaria. The period of isolation needed for subspecific differentiation

can occur in fewer than 300 generations. In a natural system involving *Plasmodium*, this could happen in as few as 60 years (19). Speculation with regard to the evolution of malaria parasites of neotropical monkeys suggests that altered selective pressures on an isolated group of mammalian malaria parasites may lead to the evolution of a new species in only a few hundred years. The time of genetic separation needed to form new species is thus generously provided within the above range.

P. vivax of the New World clearly has distinctive features that relate to its potential for spread and thus require taxonomic distinction. Justifications for the designation of subspecies are met;[1] phenotypic differences exist among parasites that occupy different sectors of the inclusive geographic range of *P. vivax.* There are subspecies of Old World *P. vivax* separated on the basis of biologic characteristics: relapse pattern (e.g., *P. vivax hibernans* and *P. vivax multinucleatum)* and morphologic characteristics such as multiple-cell invasion (20). It follows that if these were sympatric, interbreeding would have led to the loss of identity (2). The question is whether species classification is justified for the New World parasite. Historically, a major branching occurred in the *P. vivax* species, or species complex, which divided the New World *P. vivax* from known Old World parasites. The problem with either designation (species[2] or subspecies) is that it is unclear how many other taxonomic distinctions are warranted within the New World. For example, a study in Mexico links a polymorphism within the circumsporozoite protein gene with transmission and geographic distribution (21). Although this does not indicate the existence of other restricted gene pools, the circumsporozoite protein gene and transmission being genetically associated, it does show that further subdivisions are present within the New World that are being affected by mosquito distribution. We suggest that the New World parasite be given a subspecies designation, *P. vivax collins,* until it can be determined whether the New World group can be further subdivided into phenotypically defined groups occupying portions of this parasite's range. The species designation *P. collinsi* should then be considered.

Acknowledgments

We thank Margery Sullivan for help in designing the figures and critically reading the manuscript and Nancy Shulman for editorial advice and assistance.

Dr. Li was a senior postdoctoral fellow in the Laboratory of Parasitic Diseases at the National Institutes of Health during the course of this work. He is now in his second-year residency in pathology at New York University. Upon completion of his residency, he plans to continue his research efforts in infectious diseases.

References

1. Bruce-Chwatt LJ. Essential malariology. New York: John Wiley and Sons; 1985.
2. Mayr E. Animal species and evolution. Cambridge: Belknap Press of Harvard University Press; 1963.
3. Killick-Kendrick R. In: Peters W, editor. Rodent malaria. London, New York: Academic Press; 1978.
4. Collins WE, Skinner JC, Pappaioanou M, Ma NS, Broderson JR, Sutton BB, et al. Infection of *Aotus vociferans* (karyotype V) monkeys with different strains of *Plasmodium vivax*. J Parasitol 1987;73:536-40.
5. Collins WE, Warren M, Huong AY, Skinner JC, Sutton BB, Stanfill PS. Studies of comparative infectivity of fifteen strains of *Plasmodium vivax* to laboratory-reared anopheline mosquitoes, with special reference to *Anopheles culicifacies*. J Parasitol 1986;72:521-4.
6. Collins WE, McClure H, Strobert E, Skinner JC, Richardson BB, Roberts JM, et al. Experimental infection of *Anopheles gambiae s.s., Anopheles freeborni,* and *Anopheles stephensi* with *Plasmodium malariae* and *Plasmodium brasilianum*. J Am Mosq Control Assoc 1993;9:68-71.
7. Li J, Wirtz RA, McCutchan TF. Analysis of malaria parasite RNA from decade-old Giemsa-stained blood smears and dried mosquitoes. Am J Trop Med Hyg 1997;57:727-31.
8. Li J, Wirtz RA, McConkey GA, Sattabongkot J, McCutchan TF. Transition of *Plasmodium vivax* ribosome types corresponds to sporozoite differentiation in the mosquito. Mol Biochem Parasitol 1994;65:283-9.
9. Li J, Wirtz RA, McConkey GA, Sattabongkot J, Waters AP, Rogers MJ, et al. *Plasmodium*: genus-conserved primers for species identification and quantitation. Exp Parasitol 1995;81:182-90.

---

[1]The biologic diversity inherent in *P. vivax* already justifies the use of a trinomial system for naming its members that includes the designation of subspecies, a taxonomic character given formal recognition in the International Rules of Zoological Nomenclature. A subspecies is a population or group of populations inhabiting a geographic subdivision of the range of a species and differing from other populations by diagnostic morphologic characteristics.

[2]The designation of separate species does not require that the two organisms cannot mate and produce viable progeny, only that this does not happen with frequency in natural situations.

10. Wirtz RA, Burkot TR, Andre RG, Rosenberg R, Collins WE, Roberts DR. Identification of *Plasmodium vivax* sporozoites in mosquitoes using an enzyme-linked immunosorbent assay. Am J Trop Med Hyg 1985;34:1048-54.
11. Wilson RJM, Denny PW, Preiser PR, Rangachari K, Roberts K, Roy A, et al. Complete gene map of the plastid-like DNA of the malaria parasite *Plasmodium falciparum*. J Mol Biol 1996;261:155-72.
12. Waters AP, McCutchan TF. Partial sequence of the asexually expressed SU rRNA gene of *Plasmodium vivax* [published erratum appears in Nucleic Acids Res 1989 May 11;17:3630-1]. Nucleic Acids Res 17;1989;2135.
13. Escalante A, Barrio E, Ayala FJ. Evolutionary origin of human and primate malarias: evidence from the circumsporozoite protein gene. Mol Biol Evol 1995;12:616-26.
14. Vaidya AB, Morrisey J, Plowe CV, Kaslow DC, Wellems TE. Unidirectional dominance of cytoplasmic inheritance in two genetic crosses of *Plasmodium falciparum*. Mol Cell Biol 1993;13:7349-57.
15. Gupta S, Ferguson N, Anderson R. Chaos, persistence and evolution of strain structure in antigenically diverse infectious agents. Science 1998;280:912-5.
16. Lal AA, de la Cruz VF, Collins WE, Campbell GH, Procell PM, McCutchan TF. Circumsporozoite protein gene from *Plasmodium brasilianum*. Animal reservoirs for human malaria parasites? J Biol Chem 1988;263:5495-8.
17. Escalante AA, Freeland DE, Collins WE, Lal AA. The evolution of primate malaria parasites based on the gene encoding cytochrome-b from the linear mitochondrial genome. Proc Natl Acad Sci USA 1998;95:8124-9.
18. Kain KC, Brown AE, Webster HK, Wirtz A, Keystone JS, Rodriguez MH, et al. Circumsporozoite genotyping of global isolates of *Plasmodium vivax* from dried blood specimens. J Clin Microbiol 1992;30:1863-6.
19. Simpson GC. Tempo and mode in evolution. New York: Columbia University Press; 1944.
20. Coatney GR, Collins WE, Warren M, Contacos PG. The primate malarias. Bethesda: Dept of Health, Education and Welfare (US); 1971.
21. Gonzalez-Ceron L, Rodriquez MH, Nettel JC, Villarreal C, Kain KC, Hernandez JE. Differential susceptibilities of *Anopheles albimanus* and *An. pseudopunctipennis* to infections with coindigenous *Plasmodium vivax* variants VK210 and VK247 in southern Mexico. Infect Immun 1999;67:410-12.

Transferable Plasmid-Mediated Resistance to Streptomycin in a Clinical Isolate of *Yersinia pestis*

Annie Guiyoule,* Guy Gerbaud,* Carmen Buchrieser,* Marc Galimand,* Lila Rahalison,† Suzanne Chanteau,† Patrice Courvalin,* and Elisabeth Carniel*
*Institut Pasteur, Paris, France; and †Institut Pasteur, Antananarivo, Madagascar

Plasmid-mediated high-level resistance to multiple antibiotics was reported in a clinical isolate of *Yersinia pestis* in Madagascar in 1997. We describe a second *Y. pestis* strain with high-level resistance to streptomycin, isolated from a human case of bubonic plague in Madagascar. The resistance determinants were carried by a self-transferable plasmid that could conjugate at high frequencies to other *Y. pestis* isolates. The plasmid and the host bacterium were different from those previously associated with multiple-drug resistance, indicating that acquisition of resistance plasmids is occurring in this bacterial species. Emergence of resistance to streptomycin in *Y. pestis* represents a critical public health problem since this antibiotic is used as the first-line treatment against plague in many countries.

Yersinia pestis is the causative agent of plague, a disease transmitted from rodent to rodent by the bites of fleas. Bubonic plague, the most common form of the disease, occurs through rodent-to-human transmission by infected fleas of peridomestic animals (rats, cats) or wild rodents. Pneumonic plague, a less frequent but highly severe form of the disease, is transmitted from human to human by infected droplets spread by a patient with lung infection (1).

Public health measures and effective antibiotic treatments have led to a drastic decrease in plague worldwide. However, the disease is not eradicated. Endemic plague foci persist in Africa, Asia, and North and South America. During the last 15 years (1982 to 1996), 23,904 human plague cases and 2,105 deaths were reported to the World Health Organization by 24 countries (2). The most affected countries are Madagascar and Tanzania in Africa, Vietnam in Asia, and Peru in the Americas. Since the early 1990s, a steadily increasing trend in human plague cases has been reported to the World Health Organization. This trend is partly attributable to epidemics in places where human plague had disappeared for several decades and has led the World Health Organization to categorize plague as a reemerging disease (3). The reasons for plague's reemergence are not clear but may be partly attributable to inadequate surveillance.

Streptomycin, chloramphenicol, and tetracycline, alone or in combination, are the reference drugs to treat plague, whereas tetracycline or sulfonamides are recommended for prophylaxis (4). Classically, *Y. pestis* isolates are uniformly susceptible to all antibiotics active against gram-negative bacteria (5-7). Recently, high-level resistance to multiple antibiotics, including those recommended for plague prophylaxis and therapy, was observed in a clinical isolate of *Y. pestis* in Madagascar (8).

We report high-level resistance to streptomycin (the reference antibiotic for plague treatment) in a second strain of *Y. pestis* isolated in Madagascar. The resistance genes were carried by a plasmid that could conjugate at high frequencies to other *Y. pestis* isolates.

Materials and Methods

Patient and Strains

In our study of the second resistant *Y. pestis* isolate, *Y. pestis* 16/95, we used the bacterial

Address for correspondence: Elisabeth Carniel, Laboratoire des *Yersinia*, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, France; fax: 33-1-40-61-30-01; e-mail: carniel2@pasteur.fr.

strains listed in Table 1. The isolate, biotype Orientalis and ribotype Q, was obtained in 1995 in the Ampitana District of Madagascar from the axillary bubo puncture of a 14-year-old boy before antibiotic treatment. No recent history of travel outside the village was noted. Dead rats were found inside his house before the onset of the disease. The patient was treated with twice a day intramuscular injections of streptomycin (2 g per day for 4 days) and oral trimethoprim-sulfamethoxazole (2 g per day for 10 days) and recovered.

Media and Resistance Studies

Brain-heart infusion broth and agar (Difco Laboratories, Detroit, MI) were used. The MICs of antibiotics were determined on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). The cultures were incubated for 18 hours at 37°C for *Escherichia coli* and for 48 hours at 28°C for *Yersinia* strains. Aminoglycoside-modifying enzymes were assayed by the phosphocellulose paper-binding technique (13) in supernatants (centrifuged at 100,000 x *g*) after ultrasonic bacterial disintegration. Mating on filters was performed as described previously (8). Transfer frequencies were expressed as the number of transconjugants per donor colony-forming unit after the mating period. Antibiotic concentrations for selection were 100 mg/L for ampicillin, 50 mg/L for nalidixic acid, 100 mg/L for rifampicin, and 25 mg/L for streptomycin.

Nucleic Acid Techniques

Isolation of plasmid DNA, cleavage of restriction fragments, and purification of DNA fragments from agarose type VII (Sigma Chemical Co., St. Louis, MO) were performed as described elsewhere (14). Pulsed-field gel electrophoresis was performed for 18 hours with a CHEF-DRIII apparatus (Bio-Rad Laboratories, Richmond, CA), by using an electric field of 6 V/cm and an angle of 120°. Initial and final pulse times were 0.1 second and 6 seconds, respectively. Migration of the DNA fragments was performed in 0.5X Tris-Borate-EDTA buffer in a 0.9% agarose gel at 17°C. DNA sequencing reactions were performed with a Taq BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) in a Perkin-Elmer 9700 thermocycler. The samples were loaded onto 4% polyacrylamide gels and electrophoresed on a Model ABI PRISM 377 automatic DNA sequencer (Perkin-Elmer, Norwalk, CT). The nucleotide sequence of the

Table 1. Bacterial strain used in study of resistant *Yersinia pestis* isolate 16/95

Strain	Characteristics and plasmid content[a]	Source or reference
Y. pestis		
16/95	pFra, pPla, pYV, pIP1203 Tra+[b] Sm[c]	Wild strain
6/69	pFra, pPla, pYV	Wild strain
6/69cN	Nal[d], pFra, pPla	Spontaneous Nal mutant of pYV cured 6/69
6/69cNR	Nal, Rif[e], pFra, pPla	Spontaneous Rif mutant of 6/69cN
6/69cN(pIP1203)	Nal, pFra, pPla, pIP1203 Tra+ Sm	Transconjugant 16/95 x 6/69cN
Y. pseudotuberculosis		
IP32790	pYV	Wild strain
IP32790cN	Nal	Spontaneous Nal mutant of pYV cured P32790
IP32790cN(pIP1203)	Nal, pIP1203 Tra+ Sm	Transconjugant 16/95 x IP32790cN
Y. enterocolitica		
IP864	pYV	Wild strain
IP864cN	Nal	Spontaneous Nal mutant of pYV cured IP864
Escherichia coli		
C600R	*thr leuB6 thi-1 lacY supE rpoB*	Spontaneous Rif mutant of C600, Bachmann (9)
JM109	*hsdR- supE gyrA*	Yanisch-Perron et al. (10)
K802N	*hsdR- hsdM+ gal- met- supE gyrA*	Wood (11)
K802N(pIP1203)	pIP1203 Tra+ Sm	Transconjugant 16/95 x K802N

[a]Plasmid content = pFRa, pPla, and pYV are the endogenous plasmids of *Y. pestis* (12).
[b]Tra+ = self-transferable.
[c]Sm = streptomycin resistance.
[d]Nal = nalidixic acid resistance.
[e]Rif = rifampicin resistance.

strA and *strB* genes and of flanking regions from pIP1203 has been deposited in the EMBL data bank under accession number AJ249779.

Results

Streptomycin Resistance in *Y. pestis* 16/95

Disk-agar diffusion tests showed that *Y. pestis* 16/95 was resistant to streptomycin but remained susceptible to spectinomycin and other antibiotics, including those recommended for plague therapy (chloramphenicol and tetracycline) and prophylaxis (sulfonamides and tetracycline) (4). The MICs of streptomycin and spectinomycin for this strain were 1,024 mg/L and 16 mg/L, respectively. High-level resistance was due to the presence of a streptomycin phosphotransferase activity. No adenylyl transferase activity was found (data not shown).

Transfer of Streptomycin Resistance to Other Bacterial Species

Attempts were made to transfer streptomycin resistance from *Y. pestis* 16/95 by conjugation to recipient strains (MIC $\leq$ 8 mg/L) (Table 2). Transfer occurred at high frequencies (3 x 10^{-1} per donor CFU) to *Y. pestis* (MIC = 1,024 mg/L) and *Y. pseudotuberculosis* (MIC = 256 mg/L) and at lower frequencies to *E. coli* (MIC = 128 mg/L); transfer to *Y. enterocolitica* was not detected. Retransfer of streptomycin resistance from *Y. pestis* and *Y. pseudotuberculosis* transconjugants to *Y. pestis* and *Y. pseudotuberculosis* also occurred at high frequencies (3 x 10^{-2}) and was less efficient when *E. coli* was used as the recipient strain.

Characterization of Plasmid pIP1203

Plasmid DNA was extracted from *Y. pestis* 6/69 and 16/95 and digested by *Eco*RV (Figure 1A).

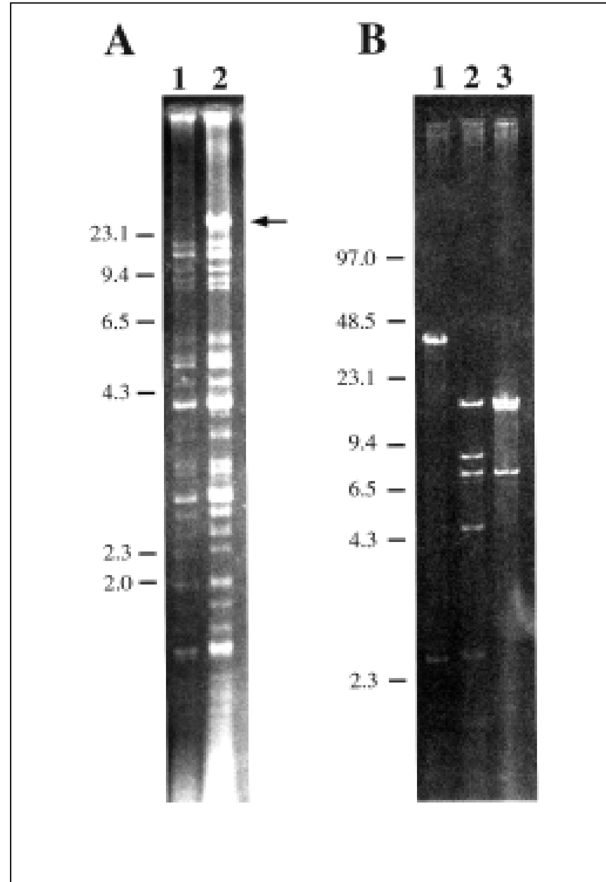


Figure 1. Analysis of plasmid pIP1203. A) Agarose-gel electrophoresis of *Eco*RV-digested plasmid DNA from representative *Yersinia pestis* strain 6/69 (1) and from streptomycin-resistant strain 16/95 (2). B) Pulsed-field gel electrophoresis of pIP1203 DNA extracted from *Escherichia coli* K802N transconjugant and digested by *Eco*RV (1), *Eco*RI+*Eco*RV (2), and *Eco*RI (3). The arrow indicates the extra large-size DNA fragment in strain 16/95. The size of the molecular weight markers in kilobases is indicated at the left of the gels.

Table 2. Conjugative transfer of pIP1203

Donor	Recipient	Frequency of transfer
Yersinia pestis 16/95	*Y. pestis* 6/69cN	3 x 10^{-1}
	Y. pseudotuberculosis IP32790cN	1 x 10^{0}
	Y. enterocolitica IP864cN	$<10^{-7}$
	E. coli K802N	2 x 10^{-5}
Y. pestis 6/69cN (pIP1203)	*Y. pestis* 6/69cNR	2 x 10^{-1}
	Y. pseudotuberculosis IP32790cNR	5 x 10^{-1}
Y. pseudotuberculosis IP32790cN(pIP1203)	*Y. pestis* 6/69cNR	3 x 10^{-2}
	Y. pseudotuberculosis IP32790cNR	2 x 10^{-2}
	E. coli C600R	$<10^{-7}$
Escherichia coli K802N (pIP1203)	*Y. pestis* 6/69cNR	6 x 10^{-2}
	Y. pseudotuberculosis IP32790cN	1 x 10^{-3}
	E. coli C600R	1 x 10^{-5}

The restriction fragments in strain 6/69 correspond to those of the three *Y. pestis* resident plasmids, pPla, pYV, and pFra (12). Comparison of the restriction profiles of strains 6/69 and 16/95 revealed that the latter contained at least one extra large-size *Eco*RV fragment corresponding to an additional plasmid, designated pIP1203. Upon transfer to *E. coli*, the size of pIP1203 was estimated (after single and double digestion with *Eco*RI and *Eco*RV) to be approximately 40 kb (Figure 1B).

Plasmid pIP1203 was stable after 100 generations in *Y. pestis* 16/95 and *E. coli* K802N (frequency of loss <0.25%). In experiments performed by reciprocal conjugation to assess the incompatibility group, pIP1203 exhibited strong incompatibility with plasmid RP4 (data not shown), which belongs to the IncP group. No incompatibility with prototype plasmids of incompatibility groups Inc FI, FII, I1, I2, N, 6-C, 7-M, 10-B-O, J, T, and W was observed (15).

Characterization of the Streptomycin-Resistance Genes

To clone the streptomycin-resistant determinant, DNA from plasmids pIP1203 and pUC18 was partially digested with *Sau*3AI and *Bam*HI, respectively, ligated, and introduced into *E. coli* JM109 (MIC of streptomycin = 2 mg/L). The smallest recombinant plasmid conferring streptomycin resistance, pAT709, contained an 11-kb insert. The resistance determinant was subcloned by introducing a 2.7-kb *Hin*cII fragment of the 11-kb insert into pUC18, which generated pAT710. This recombinant plasmid conferred high levels of resistance to the new host (MIC of streptomycin = 512 mg/L) by synthesis of a streptomycin phosphotransferase. Sequence determination of the insert in pAT710 revealed that the base composition of this fragment was 57.5% G+C, much higher than the average G+C content of the *Y. pestis* genome (46%) (16). Two potentially coding sequences of 801 bp and 834 bp identified in the insert were identical to the *strA* and *strB* genes that encode an aminoglycoside 3"-*O*-phosphotransferase [APH(3")-Ib] and a 6-*O*-phosphotransferase [APH(6)-Id], respectively (17). The *str* genes were originally described in the small, nonconjugative, broad-host-range IncQ plasmid RSF1010 (18). They have been subsequently found as part of transposon Tn*5393* and related elements in phytopathogenic *Erwinia amylovora*, *Pseudomonas syringae* pv. papulans, and *Xanthomonas campestris* pv. vesicatoria (19,20).

An 81-bp sequence identical to the inverted terminal repeat (IR) of Tn*5393* was identified downstream from pIP1203 *strA*-*strB* genes (Figure 2). This IR is always present at the same position in the various genetic structures that

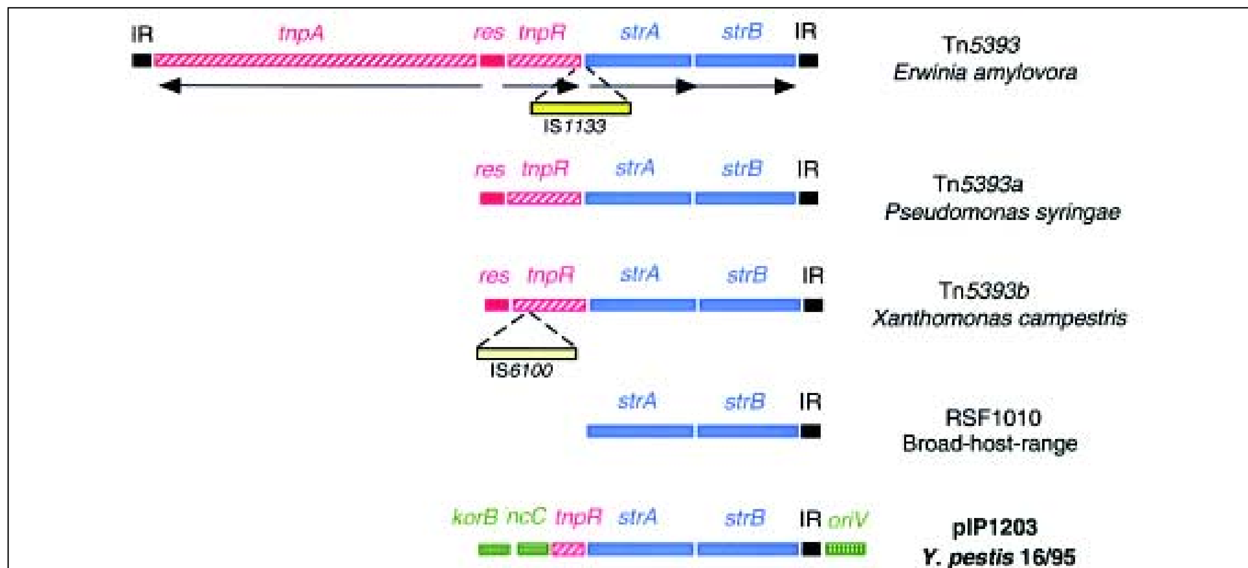


Figure 2. Genetic organization of the *strA*-*strB* genes. Schematic representation of the regions of Tn*5393* and derivatives and of plasmids RSF1010, and pIP1203 carrying the *strA* and *strB* genes. IR, inverted repeat; *tnpA*, transposase; *res*, resolution site; *tnpR*, resolvase; IS*1133* and IS*6100*, insertion sequences; *korB* and *incC*, genes homologous to those involved in regulation and partition of plasmid R751, respectively; *oriV*, origin of vegetative replication of R751. Direction of gene transcription is indicated by arrows.

carry the *str* genes. Upstream from *strA*, the sequence was identical to a portion of the *tnpR* resolvase-repressor gene of Tn*5393*, Tn*5393*a, and Tn*5393*b (20). The identity was interrupted after 105 bp within the 3'-end of *tnpR* (Figure 2). Like Tn*5393*a, pIP1203 possessed the TAG motif, which represents a putative insertion target for IS*1133* (20).

The *tnpR*-*strA*-*strB*-IR region of pIP1203, which displayed a Tn*5393*-like organization, was flanked on both sides by sequences highly similar to portions of the broad-host-range IncP plasmid R751 (21). Upstream from the truncated *tnpR* gene, there was identity with the 3'-end of the *incC2* and the 5'-end of the *korB* genes [positions 3396 to 3820 of plasmid R751, numbering according to GenBank accession number U67194]. Downstream from the IR, identity was found with a portion of plasmid R751 (positions 9796 to 9947, numbering according to GenBank accession number U67194), located in the vicinity of the *oriV* vegetative origin of replication.

Discussion

Y. pestis strain 16/95, isolated in Madagascar in 1995 from a human case of bubonic plague, carried the self-transferable plasmid pIP1203 conferring resistance to streptomycin. The strain of *Y. pestis* 17/95 harboring the multidrug-resistance conjugative plasmid pIP1202 described in 1997 (8) was also isolated in Madagascar from a human case. However, the two strains differ in several aspects: they were isolated in two districts of Madagascar (Ambalavao and Ampitana) that are 120 km (80 miles) apart; strain 17/95 is of the typical ribotype B, whereas strain 16/95 is of the newly described, Madagascar-specific, ribotype Q (22); plasmid pIP1202 carries multiple antibiotic resistance genes, belongs to the Inc6-C group, and is 150 kb in size, whereas pIP1203 carries only the streptomycin resistance determinants, belongs to the IncP group, and is 40 kb in size; and streptomycin resistance is due to adenylylation of the drug in strain 17/95 and to phosphorylation in strain 16/95. Therefore, the two resistant *Y. pestis* isolated in Madagascar correspond to distinct strains that have acquired different conjugative plasmids.

The streptomycin resistance genes in pIP1203 are part of the *tnpR*-*strA*-*strB*-IR cluster characteristic of the Tn*5393* group of transposons. This portion of the element is inserted in R751, a broad-host-range plasmid belonging to the IncP group. The sequences flanking the *tnpR*-*strA*-*strB*-IR region in pIP1203 are separated by approximately 6 kb in the original R751 backbone (21). This organization suggests that insertion of a Tn*5393*-like element was associated with concomitant (or subsequent) loss of a region involved in the control of plasmid stability. Despite this deletion, pIP1203 appears to be highly stable in both *Y. pestis* and *E. coli*.

IncP plasmids are promiscuous; therefore, the original host of pIP1203 remains unknown. However, since this plasmid was extremely stable in *Y. pestis* 16/95, conferred high-level resistance to streptomycin, and could transfer at remarkably high frequencies to other strains of *Y. pestis*, it is possible that pIP1203 was acquired a long time ago and is now well adapted to this bacterial species.

It is not known where genetic transfer of the resistance plasmid took place. During its flea-host-flea cycle, *Y. pestis* may have been in contact with the donor cell, either in its mammalian host (rodent or human) or the insect vector. In mammals, *Y. pestis* circulates in a usually sterile milieu (lymphatic vessels, spleen, liver, blood, and sometimes lungs). Contact with the bacterial donor and transfer of pIP1203 may have occurred in the bloodstream at the premortem stage of infection, when gut bacteria invade the host. Alternatively, plasmid acquisition may have taken place in the midgut of the flea, a nonsterile environment where *Y. pestis* is most likely to be in intimate contact with other microorganisms.

From a clinical and public health point of view, this report is of great concern and indicates that surveillance of antibiotic resistance in *Y. pestis* should become systematic worldwide. Streptomycin, an inexpensive, easy to use, and highly effective drug against *Y. pestis,* represents the therapy of choice for plague in Madagascar. Spread of plasmids pIP1202 and pIP1203 among strains of *Y. pestis* would render streptomycin ineffective for plague treatment and could create economic and therapeutic problems in Madagascar and other countries with endemic plague foci.

This work was supported in part by a Bristol-Myers Squibb Unrestricted Biomedical Research Grant in Infectious Diseases.

A. Guiyoule is a research technician. Her research interests are bacterial pathogenesis and antibiotic resistance.

References

1. Pollitzer R. Plague. In: WHO Monograph Series 22 World Health; Geneva: World Health Organization; 1954.
2. World Health Organization. Human plague in 1996. Wkly Epidemiol Rec 1998;73:366-9.
3. Schrag SJ, Wiener P. Emerging infectious diseases: what are the relative roles of ecology and evolution? Trends Evol Ecol 1995;10:319-24.
4. Barnes AM, Quan TJ. Plague. In: Gorbach SL, Bartlett JG, Blacklow NR, editors. Infectious diseases. Philadelphia: W. B. Saunders Company; 1992:1285-91.
5. Rasoamanana B, Leroy F, Raharimanana C, Chanteau S. Surveillance de la sensibilité aux antibiotiques des souches de *Yersinia pestis* à Madagascar de 1989 à 1995. Arch Inst Pasteur Madagascar 1995;62:108-10.
6. Frean JA, Arntzen L, Capper T, Bryskier A, Klugman KP. In vitro activities of 14 antibiotics against 100 human isolates of *Yersinia pestis* from a southern African plague focus. Antimicrob Agents Chemother 1996;40:2646-7.
7. Smith MD, Vinh DX, Hoa NTT, Wain J, Thung D, White NJ. In vitro antimicrobial susceptibilities of strains of *Yersinia pestis*. Antimicrob Agents Chemother 1995;39:2153-4.
8. Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, et al. Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. N Engl J Med 1997;337:677-80.
9. Bachman B. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In: Neidhardt F, editor. *Escherichia coli* and *Salmonella*, cellular and molecular biology, 2nd ed. Washington, DC: ASM Press; 1996:2460-88.
10. Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 1985;33:103-19.
11. Wood WB. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. J Mol Biol 1966;16:118-33.
12. Ferber DM, Brubaker RR. Plasmids in *Yersinia pestis*. Infect Immun 1981;31:839-41.
13. Haas MJ, Dowding JE. Aminoglycoside-modifying enzymes. Methods Enzymol 1975;43:611-28.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1989.
15. DNA insertion elements, plasmids, and episomes. In: Bukhari AI, Shapiro JA, Adhya SL, editors. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1977. p. 601-38.
16. Bercovier H, Mollaret HH, Alonso JM, Brault J, Fanning GR, Steigerwalt AG, et al. Intra- and interspecies relatedness of *Yersinia pestis* by DNA hybridization and its relationship to *Yersinia pseudotuberculosis*. Curr Microbiol 1980;4:225-9.
17. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev 1993;57:138-63.
18. Scholz P, Haring V, Wittmann-Liebold B, Ashman K, Bagdasarian M, Scherzinger E. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene 1989;75:271-88.
19. Chiou CS, Jones AL. Nucleotide sequence analysis of a transposon (Tn*5393*) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. J Bacteriol 1993;175:732-40.
20. Sundin GW, Bender CL. Expression of *strA-strB* streptomycin resistance genes in *Pseudomonas syringae* and *Xanthomonas campestris* and characterization of IS*6100* in *X. campestris.* Appl Environ Microbiol 1995;61:2891-7.
21. Thorsted PB, Macartney DP, Akhtar P, Haines AS, Ali N, Davidson P, et al. Complete sequence of the IncPß plasmid R751: implications for evolution and organisation of the IncP backbone. J Mol Biol 1998;282:969-90.
22. Guiyoule A, Rasoamanana B, Buchrieser C, Michel P, Chanteau S, Carniel E. Recent emergence of new variants of *Yersinia pestis* in Madagascar. J Clin Microbiol 1997;35:2826-33.

Nested Polymerase Chain Reaction for Amplification of the *Cryptosporidium* Oocyst Wall Protein Gene

Susana Pedraza-Díaz,* Corinne Amar,*
Gordon L. Nichols,† Jim McLauchlin*
*PHLS Central Public Health Laboratory, London, UK;
†PHLS Communicable Disease Surveillance Centre, London, UK

We developed a sensitive nested polymerase chain reaction procedure for the *Cryptosporidium* oocyst wall protein (COWP) gene. Amplification and genotyping were successful in 95.2% of 1,680 fecal samples, 77.6% by the unnested and 17.6% by the nested COWP procedure. The COWP gene was amplified from 2,128 fecal samples: 71 from livestock animals and 2,057 from humans. This series included 706 cases from seven drinking water-associated outbreaks and 51 cases from five swimming pool-associated outbreaks, as well as 1,300 sporadic cases.

The coccidian parasite *Cryptosporidium* is now recognized as a major cause of waterborne diarrheal disease worldwide (1,2). The exact modes of transmission, however, are often unclear, and the relative importance of foreign travel; consumption of foods, beverages, or water; person-to-person transmission; and infected animals in disease transmission remain to be ascertained (1,2).

Characterization of *Cryptosporidium parvum* by phenotypic and genotypic methods (3-9) has identified two major types associated with human infection: one exclusively from humans and a single nonhuman primate (genotype 1 or human type) and a second in livestock as well as humans (genotype 2 or calf type). Experimental infection of both calves and mice was successful with genotype 2 but not with genotype 1 (4). Polymorphic genes that identify these genotypes include the *Cryptosporidium* oocyst wall protein (COWP) gene (5), the thrombospondin-related adhesive proteins *Cryptosporidium*-1 (TRAP-C1 [6]), and *Cryptosporidium*-2 (TRAP-C2 [4]). These observations concerning the two genotypes of *C. parvum* may reflect the epidemiology of two parasites with distinct and exclusive transmission cycles (4,9) and may represent two species (8).

Address for correspondence: J. McLauchlin, Food Safety Microbiology Laboratory, PHLS Central Public Health Laboratory, 61 Colindale Avenue, London, NW9 5HT, UK; fax: +44 20 8200 8264; e-mail: jmclauchlin@phls.nhs.uk.

We have described the identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of a single human isolate with an unusual COWP genotype, designated genotype 3 (10). Several *Cryptosporidium* species are associated with human disease, including *C. felis*, *C. meleagridis*, and an as-yet-unnamed species designated the dog type (11,12). DNA sequencing of multiple genes from six human isolates of COWP genotype 3 indicates that separate species status is justified; its 18S rDNA sequences are identical to those of *C. meleagridis* (13). Since the host range of the various *Cryptosporidium* species and *C. parvum* genotypes infectious to humans differs, their epidemiology is also likely to differ.

We have described a simple DNA extraction method from whole feces, suitable for amplification of *Cryptosporidium* DNA, and have applied it to 397 cryptosporidiosis cases, including sporadic human and animal cases as well as cases from two large waterborne outbreaks (8,10). In 218 sporadic human cases, DNA could not be amplified from 9% of samples for genotyping by PCR-RFLP analysis of the COWP gene (5,8), despite amplification of 18S rDNA fragments or detection of oocysts by microscopy. The purposes of this study were to develop sensitive methods for identifying *Cryptosporidium* genotypes in DNA extracted from whole feces and to assess the application of these techniques to large numbers of samples.

Materials and Methods

Fecal Samples, Oocyst Disruption, and DNA Extraction

Whole feces were collected from naturally infected humans and livestock animals; the samples contained *Cryptosporidium* oocysts morphologically indistinguishable from *C. parvum* by light microscopy (8,14). One sample of feces from a sheep experimentally infected with a standard (Moredun) strain originally of cervine origin (15) was included. Human feces were also tested in which no *Cryptosporidium* was detected but *Cyclospora* oocysts or *Giardia* cysts were detected by conventional techniques. All specimens were stored at 4°C without preservatives. Oocyst disruption and DNA purification were performed (8).

Staining and Light Microscopy

Samples were reexamined by light microscopy after being stained by the modified Ziehl-Neelsen (MZN) acid-fast method (14) and the immunofluorescence (IF) method (8) with an anti-*Cryptosporidium*-oocyst monoclonal antibody designated MAB-C1 (16).

PCR-RFLP analysis

PCR of two 18S rDNA fragments (reaction 1 [8,17] and reaction 2 [18]), COWP (5), TRAP-C1 (6), and TRAP-C2 (4) gene fragments, as well as restriction digestion with *Rsa*I for the COWP and TRAP-C1 genes, was performed. The 18S rDNA reaction 2 (18) was modified as described by Bornay-Llinares et al. (19) to include 4 mM $MgCl_2$, with a program cycle of 95°C for 5 min, 45 cycles of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min, followed by a final extension at 72°C for 9 min.

For the nested-COWP procedure (N-COWP), a 769-bp fragment of the COWP gene was amplified with primers BCOWPF (5'-ACCGCT TCTCAACAACCATCTTGTCCTC-3') and BCOWPR (5'-CGCACCTGTTCCCACTCAATGTA AACCC-3'), which encompasses the 553-bp fragment (5). Primers BCOWPNF and BCOWPNR were designed by using the PRIME program in the Genetics Computer Group Wisconsin package (Madison, WI). PCR amplification was performed in 25-µl volumes with 2.5 µl of DNA in 1x PCR buffer, 1.5 mM $MgCl_2$, 250 µM of each dNTP, 10 pmoles of each primer, and 1.25 units of Taq DNA polymerase. Samples were subjected to 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. This single reaction is referred to as the extended-COWP (E-COWP) reaction. For the N-COWP procedure, a 553-bp gene fragment was then amplified from 2.5 µl of the E-COWP material as described (5), except that each primer (Cry9 and Cry15) was used at 10 pmoles. Positive and negative controls for all PCR procedures were included at all stages and for all batches.

For the N-COWP, E-COWP, COWP, 18S rDNA 1, 18S rDNA 2, TRAP-C1, and TRAP-C2 gene fragments, 5-µl aliquots of the PCR products were analyzed by electrophoresis in 1% agarose-ethidium bromide gels. *Rsa*I digestion of N-COWP, COWP, and TRAP-C1 fragments was resolved by electrophoresis in 3.2% typing-grade agarose gels containing ethidium bromide. All gels were recorded by using UV transillumination and Polaroid Type 667 film.

DNA Sequencing

PCR products were purified in Microspin S-400 HR (Pharmacia Biotech, St. Albans, UK) and cloned by using the TOPO-TA Cloning kit (Invitrogen, Groningen, the Netherlands). Plasmid DNA was purified by using the Promega Wizard SV kit (Promega, Madison, WI), and clones were sequenced on an ABI377 automated sequencer with BigDye terminator chemistry with the M13(-21) primer at the Single Reaction DNA Sequencing Service (Cambridge Bioscience Ltd., Cambridge, UK). Sequences were analyzed with the Genetics Computer Group (GCG) program package (University of Wisconsin, Madison, WI).

Results

Nested COWP Procedure

Analysis of the published genotype 2 COWP gene sequence (GenBank accession numbers Z22537) led to design of two primers (BCOWPF and BCOWPR) to amplify a predicted E-COWP 769-bp fragment, which includes the 553-bp fragment amplified by the previously described Cry15/Cry9 primers (5). Appropriately sized fragments were generated by using the BCOWPF and BCOWPR primer pair from DNA extracted from human fecal samples containing *Cryptosporidium* genotypes 1, 2, and 3. The three respective amplicons were cloned and sequenced; the sequences are available from GenBank:

accession numbers af248741 (genotype 1), af248743 (genotype 2), and af248742 (genotype 3). Identical sequences were obtained from the genotype 2 sequence (accession number Z22537), with the exception of the insertion of three nucleotides.

The N-COWP amplification procedure for the 553-bp COWP fragment from the 769-bp E-COWP amplicon was developed and initially assessed by using 76 DNA samples extracted from whole human feces, which had been genotyped by using the COWP reaction. Identical results were obtained by the COWP and N-COWP procedures: 28 were genotype 1, 34 genotype 2, and 5 genotype 3; both genotypes 1 and 2 were recovered from nine samples. No amplicons were detected by the N-COWP procedure with DNA extracted from *Toxoplasma gondii* tachyzoites (two samples), *Eimeria tenella* oocysts (two samples), and feces containing either *Cyclospora* oocysts (10 samples) or *Giardia* cysts (11 samples).

N-COWP by Different PCR Procedures

To assess the sensitivity of PCR procedures for the N-COWP reaction, amplification of DNA was compared with that from the E-COWP, COWP, TRAP-C1, and TRAP-C2, as well as the two 18S rDNA reactions. DNA samples prepared from whole feces were decimally diluted in water to 10^{-4}, and each dilution was tested by all procedures. Samples were prepared from human feces containing genotypes 1, 2, or 3 and from ovine feces (Moredun strain) containing genotype 2 (Table 1).

The N-COWP reaction strongly amplified DNA from all samples to a dilution of 10^{-3}, with the exception of the genotype 3 sample, in which

Table 1. Sensitivities of different polymerase chain reaction procedures for *Cryptosporidium* gene sequences

Dilutions of	PCR procedure[a]						
DNA sample	N-COWP	E-COWP	COWP	TRAP-C1	TRAP-C2	18S rDNA 1	18s rDNA 2
Human, genotype 1							
Undiluted	+++[b]	++	++	++	++	++	++
10^{-1}	+++	++	++	++	++	++	++
10^{-2}	+++	+	++	—	+	+	++
10^{-3}	+++	—	—	—	—	+	++
10^{-4}	—	—	—	—	—	—	—
Human, genotype 2							
Undiluted	+++	++	++	++	++	++	++
10^{-1}	+++	+	++	+	—	++	++
10^{-2}	+++	—	+	+	—	+	++
10^{-3}	+++	—	—	—	—	±	±
10^{-4}	—	—	—	—	—	—	—
Human, genotype 3 (*Cryptosporidium meleagridis*)							
Undiluted	+++	+	+	+	—	+	++
10^{-1}	+++	±	—	—	—	+	±
10^{-2}	+++	—	—	—	—	—	—
10^{-3}	—	—	—	—	—	—	—
10^{-4}	—	—	—	—	—	—	—
Ovine, genotype 2 (Moredun strain)							
Undiluted	+++	++	++	++	++	++	++
10^{-1}	+++	+	++	+	+	++	++
10^{-2}	+++	±	±	—	—	+	++
10^{-3}	+++	—	—	—	—	—	±
10^{-4}	—	—	—	—	—	—	—

[a]Polymerase chain reaction (PCR) procedures used for gene fragments: N-COWP = nested *Cryptosporidium* oocyst wall protein gene (553 bp, this study); E-COWP = extended COWP (769 bp, this study); COWP = unnested procedure (553 bp, 5); TRAP-C1 = thrombospondin-related adhesive protein *Cryptosporidium* 1 (506 bp, 6); TRAP-C2 = thrombospondin-related adhesive protein *Cryptosporidium* 2 (369 bp, 4); 18S rDNA 1 (422 bp, 8,17); 18S rDNA 2 (435 bp, 18).

[b]Semiquantitative results on the basis of strength of ethidium bromide staining in electrophoresis gels: strong (+++), moderate (++), weak (+), very weak (±), and amplification not detected(—).

amplification was achieved only up to 10^{-2} (Table 1). The two 18S rDNA procedures moderately amplified DNA to 10^{-3}, with the exception of genotype 3, which gave only a weak reaction in the undiluted sample. The three PCRs used for genotyping (COWP, TRAP-C1, and TRAP-C2) all gave weaker signals than the N-COWP procedure, and product was sufficient for restriction enzyme digestion undiluted or at 10^{-1}, except for genotype 3, in which there was insufficient amplification with all reactions, and COWP, in which there was sufficient amplification from the sample containing genotype 1 to perform genotyping at the 10^{-2} dilution (Table 1).

Assessment of N-COWP Procedure

DNA was extracted from 1,680 fecal samples in which hospital laboratories had reported detection of *Cryptosporidium* oocysts by conventional procedures; these samples were from patients with diarrhea diagnosed in England, Northern Ireland, or Scotland during 1998-99. All samples were tested by the unnested COWP procedure, and those in which no amplicons were detected were retested by N-COWP. Samples were reexamined by microscopy if no amplification was detected by either COWP and N-COWP (except for two samples for which there was insufficient material) and a selection of other samples: overall, 475 (28%) and 397 (24%) of samples were retested by IF and MZN, respectively. Amplification and genotyping were successful in 95.2% of the samples, 77.6% by COWP and 17.6% by N-COWP (Table 2). Of the 43 samples in which no oocysts were detected, all were negative by COWP, N-COWP, and 18S rDNA-1 PCR. DNA was amplified from two of the 43 microscopy-negative samples by the 18S rDNA-2 reaction. Five of these microscopy-negative samples did not amplify DNA when tested with TRAP-C1.

Of the 35 COWP- and N-COWP-negative samples in which oocysts were detected after reexamination (Table 2), DNA was amplified from 11 (31%) by either 18S rDNA amplifications: three and four samples by 18S rDNA reactions 1 and 2, respectively, and four samples by both 18S rDNA amplifications.

Of the 1,600 samples in which DNA was amplified by either COWP or N-COWP (Table 2), 731, 209, and 210 were also tested by 18S rDNA reaction 1, 18S rDNA reaction 2, and TRAP-C2, respectively. DNA was amplified from 627 (86%), 166 (79%), and 138 (66%) by 18S rDNA reaction 1, 18S rDNA reaction 2, and TRAP-C2, respectively. Identical genotyping results were obtained by COWP/N-COWP and *Rsa*1 digestion of the TRAP-C1 fragment in all 138 samples in which amplification of the latter DNA fragment was achieved: 55 were genotype 1 and 83 genotype 2. The proportions of genotype 1 and genotype 2 amplifications were similar by COWP or N-COWP; however, there was a greater than tenfold increase in the proportions of both mixed genotypes 1 and 2 and genotype 3 detection by N-COWP (Table 2).

COWP and N-COWP and Epidemiologic Studies

The COWP gene was amplified from 2,128 cryptosporidiosis cases: 71 from livestock animals and 2,057 from humans (Table 3). Among the samples from humans, a genotype was established by N-COWP but not by COWP in 476 (23.1%) of 2,057, 253 (35.8%) of 706, 13 (25.5%) of 51, and 210 (16.2%) of 1,300 of all samples, and those collected from drinking waterborne

Table 2. *Cryptosporidium* oocyst wall protein (COWP) gene analysis of DNA extracted from 1,680 human fecal samples

PCR amplication procedure	No. of samples (%)	COWP genotypes 1	2	1 & 2	3
COWP gene fragment amplified					
Unnested	1,304 (77.6)	381	917	2	4
Nested[a]	296 (17.6)	81	198	7	10
COWP gene fragment not amplified[b]					
Oocysts detected by microscopy	35 (2.1)				
Microscopy not reconfirmed[c]	2 (0.1)				
Oocysts not reconfirmed by microscopy	43 (2.6)				

[a]All samples previously negative by unnested procedure.
[b]By both unnested and nested procedures.
[c]Insufficient material available.
PCR = Polymerase chain reaction.

Table 3. *Cryptosporidium* oocyst wall protein (COWP) gene analysis of DNA from 2,057 humans and 71 livestock animals

	COWP genotypes				
	1	2	1 & 2	3	Reference
Humans					
2,057 cases	795	1,227	20	15	This study
Drinking water-associated outbreaks					
1	140	2	3	0	20,21
2	158	14	1	1	22
3	4	0	0	0	20
4	15	0	0	0	23
5	0	6	0	0	24
6	0	25	0	0	24
7	4	331	0	2	24
Swimming pool-associated outbreaks					
8	3	0	0	0	24
9	6	3	0	0	25
10	0	10	0	0	25
11	9	1	3	0	25
12	14	2	0	0	25
Sporadic human cases	442	833	13	12	8, this study
Livestock animals					
Calves, lambs	0	71	0	0	This study

outbreaks, swimming-pool outbreaks, and sporadic cases, respectively. Of samples from livestock animals, 10 (14.1%) of 71 genotypes were established by N-COWP but not COWP. Genotype 1 was found in 38.6% of the human samples, genotype 2 in 59.6%, both genotype 1 and 2 were detected in 1.0%, and genotype 3 (*C. meleagridis*) in 0.7%.

Genotyping results were obtained from 706 patients infected during seven drinking water-associated outbreaks: genotype 1 was predominantly recovered from patients in outbreaks 1 to 4, and genotype 2 from most of the patients in outbreaks 5 to 7 (Table 3). Genotyping results were obtained from 51 patients during five swimming pool-associated outbreaks (Table 3). Two of these outbreaks (8 and 10) were due to a single genotype, and the remaining three (9, 11, and 12) involved both genotypes 1 and 2 (Table 3). Two samples from swimming pool outbreak 6, which were from a single patient, yielded genotype 1 at first and both genotypes 1 and 2 six days later. Of 1,300 sporadic cases, 34.0% were genotype 1, 64.1% genotype 2, 1% were both genotypes 1 and 2, and 9% were genotype 3 (*C. meleagridis*).

Conclusions

Human cryptosporidiosis has multiple potential host reservoirs of infection and multiple routes of transmission (1,2). Molecular biologic methods have allowed identification of two major genotypes within *C. parvum* (the principal infectious agent for human cryptosporidiosis) with two transmission cycles. The application of genotyping techniques may therefore provide a better understanding of the epidemiology of cryptosporidiosis, including different routes of transmission.

Epidemiologic studies of cryptosporidiosis have incorporated results from genotyping *C. parvum* (4,26-29), although these have been applied to relatively few samples. For example, among the estimated 400,000 cases associated with the 1993 waterborne outbreak in Milwaukee (30), genotyping data are available for five patients (all genotype 1 [4,26]). However, *C. parvum* genotype 1 was implicated in outbreaks associated with drinking and food, as well as person-to-person transmission in a day-care center and attendance at a water park (4,26-29). *C. parvum* genotype 2 was also associated with waterborne outbreaks, contaminated apple juice, and contact with cows (4,26,27). To investigate the epidemiology of cryptosporidiosis, we have described simple methods for the extraction of cryptosporidium DNA from whole feces and applied genotyping techniques to several hundred samples (8,10). We applied these techniques, together with the development and

application of a sensitive PCR protocol (N-COWP), to >2,000 samples. Our techniques are less labor intensive than other methods (11,26,27) and allow analysis of large numbers of samples: we estimate that 1,000 samples can be extracted and genotyped within 6 months by one scientist working full time.

The N-COWP genotyping protocol is more sensitive than three unnested procedures (COWP, TRAP-C1, and TRAP-C2) also used for genotyping. The higher copy number of the 18S rDNA genes means that PCR procedures for these are likely to be more sensitive than those for the COWP, TRAP-C1, and TRAP-C2 gene sequences, and our data are consistent with this observation: the 18S rDNA genes occur as five copies (31), but the COWP, TRAP-C1 and TRAP-C2 genes occur as single copies per genome (32). Nested procedures for a single copy gene (the dihydrofolate reductase gene) and 18S rDNA genes were most sensitive when 11 PCR techniques for genotyping of *Cryptosporidium* were compared, although these studies were performed on DNA extracted from four semipurified oocyst suspensions (33).

One of the 18S rDNA amplifications reported elsewhere for genotyping (33) also amplified DNA from different *Cryptosporidium* species. However, Sulaiman and colleagues (33) reported that a COWP gene can be amplified from *C. serpentis* and *C. muris* (although the PCR products were faint) and that these are distinct from *C. parvum*. *C. wrairi* (5) and *C. meleagridis* (34) can be distinguished by PCR-RFLP analysis. We also reported that a single base mismatch (T to C substitution) occurs in the Cry9 COWP primer annealing region in genotype 3 (*C. meleagridis*) (13), which may account for the increased efficiency in amplification with the N-COWP procedure, as well as the faint amplifications reported for *C. serpentis* and *C. muris* (33). We are investigating the use of our extraction and PCR protocols described for identification of *Cryptosporidium* species, especially in samples that did not amplify COWP sequences but did amplify cryptosporidium 18S rDNA and in which oocysts were detected by microscopy.

A diagnosis of cryptosporidiosis can be established by examination of stained fecal smears prepared either directly from feces or after concentration (flotation) procedures (14). Although symptomatic cryptosporidiosis in humans is generally associated with large numbers of oocysts in the feces, infections occur in which oocysts cannot be detected by microscopy (14,35). Our DNA extraction method is based on whole feces; therefore, target DNA may be derived not only from oocysts, but also from other stages in the life cycle of this parasite. However, as found in experiments seeding DNA into feces, oocysts are the most likely source of DNA and the estimated sensitivity of the N-COWP reaction is equivalent to <500 oocysts/g of feces (Pedraza-Díaz et al., unpub data). Future studies will examine specimens from patients with diarrhea due to *Cryptosporidium* (and other intestinal pathogens) to establish the true sensitivity of this method for patient samples without detectable oocysts. Failure to detect oocysts may result from degradation of both oocysts and DNA, although DNA has been isolated and successfully amplified from whole fecal samples stored at 4ºC for >5 years (8).

The N-COWP procedure detected a higher proportion of samples containing both genotypes 1 and 2. Further DNA sequence-based analysis indicates that these are true dual infections, not infections due to recombination within *C. parvum* (Pedraza-Díaz et al., unpub data). The increased rate of mixed infections identified by the N-COWP procedure is consistent with our data suggesting that the two genotypes may occur in feces at differing concentrations (8). Previously undetectable mixtures of genotypes may also explain apparent genetic changes due to selective growth as a result of host specificity after passage through different animals (7,36).

In this large series of cryptosporidiosis cases, all samples from livestock yielded genotype 2, consistent with previous results (9). Of >2,000 samples from humans, 38.6% were due to genotype 1, 59.6% to genotype 2, both genotypes 1 and 2 were recovered from 1%, and 0.7% were due to genotype 3 (*C. meleagridis)*. There are relatively few comparative data analyzing larger series from humans, although Sulaiman et al. (26) reported that of 50 human cases, 82% were due to genotype 1 and 18% to genotype 2. These results with respect to the proportions of the *C. parvum* genotypes 1 and 2 differed markedly from our data for the United Kingdom; however, further results showed marked seasonal and geographic differences (34).

Data are presented here on 709 patients infected during seven drinking-waterborne outbreaks (51% of the microbiologically confirmed

cases). Four of the outbreaks were almost exclusively due to genotype 1 and three to genotype 2. Data from field epidemiologic observations (23,24) suggest that contamination of water by sheep feces was involved in the three outbreaks due to genotype 2. All outbreaks occurred in the spring when lambing (as well as outbreaks of cryptosporidiosis in sheep) occurs most commonly in the United Kingdom (37). In contrast, the likely source of *C. parvum* in the four drinking-waterborne outbreaks predominantly due to genotype 1 was by contamination with human sewage; these occurred throughout the year. Outbreaks 1 and 2 occurred after heavy rain (20-22), and untreated sewage overflowing from storm drains may be a contributing factor.

Among the five outbreaks associated with swimming pools, one was due to genotype 1, one to genotype 2, and the remaining three to both genotypes 1 and 2. Outbreaks in swimming pools may be associated with fecal contamination from a single infected person (especially in toddler pools), so that a single genotype is recovered from the patients. However, outbreaks may also be due to more general problems such as contamination with sewage, poor disinfection, or inadequate maintenance of filtration equipment (25).

Our data on 1,300 sporadic cases, as well as further epidemiologic analysis (34), indicate that patients reporting contact with animals or farms were almost all infected by genotype 2; the spring peak in cases was almost exclusively due to genotype 2; genotype 1 was significantly more common in patients infected during the late summer-autumn peak and in those with a history of foreign travel; and there were distinct geographic variations in the distribution of the genotypes.

In summary, we described methods for the analysis of *Cryptosporidium* genotypes and demonstrated their application to a large series of samples. These approaches, together with the development of more discriminatory typing methods (28), should increase understanding of the epidemiology of cryptosporidiosis. Methods of improved sensitivity, such as those described here, will also be invaluable in detection and genotyping of *Cryptosporidium* in environmental samples.

Acknowledgment

We thank colleagues in clinical microbiology laboratories for the donation of specimens.

The work of Dr. Pedraza-Díaz is funded by Biomed Grant PL 962557 from the European Commission and is jointly supervised by the Public Health Laboratory Service and the Imperial College of Science and Technology and Medicine, London, United Kingdom.

Dr. Pedraza-Díaz is a research student in the Central Public Health Laboratory of the PHLS. Her work focuses on the development of molecular biological methods for the study of intestinal infectious diseases.

References

1. Fayer R, Speer CA, Dubey JP. The general biology of *Cryptosporidium*. In: Fayer R, editor. *Cryptosporidium* and cryptosporidiosis. Boca Raton: CRC Press; 1997. p. 1-41.
2. Meinhardt PL, Casemore DP, Miller KB. Epidemiological aspects of human cryptosporidiosis and the role of waterborne transmission. Epidemiol Rev 1996;18:118-36.
3. Awad-el-Kariem FM, Robinson HA, Dyson DA, Evans D, Wright S, Fox MT, et al. Differentiation between human and animal strains of *Cryptosporidium parvum* using isoenzyme typing. Parasitology 1995;110:129-32.
4. Peng MM, Xiao L, Freeman AR, Arrowood MJ, Escalante AA, Weltman AC, et al. Genetic polymorphisms among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. Emerg Infect Dis 1997;3:567-73.
5. Spano F, Putignani L, McLauchlin J, Casemore DP, Crisanti A. PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum* isolates of human and animal origin. FEMS Microbiol Lett 1997;150:209-17.
6. Spano F, Putignani L, Guida S, Crisanti A. *Cryptosporidium parvum*: PCR-RFLP analysis of the TRAP-C1 (thrombospondin-related adhesive protein of *Cryptosporidium*-1) gene discriminates between two alleles differentially associated with parasite isolates of animal and human origin. Exp Parasitol 1998;90:195-8.
7. Widmer G. Genetic heterogeneity and PCR detection of *Cryptosporidium parvum*. Adv Parasitol 1998;40:223-39.
8. McLauchlin J, Pedraza-Díaz S, Amar-Hoetzeneder C, Nichols GL. Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. J Clin Microbiol 1999;37:3153-8.
9. Morgan UM, Xiao L, Fayer R, Lal AA, Thompson RCA. Variation within *Cryptosporidium*: towards a taxonomic revision of the genus. Int J Parasitol 1999;29:1733-51.
10. Patel S, Pedraza-Díaz S, McLauchlin J, Casemore DP, Outbreak Control Team South and West Devon 1996, Incident Management Team and Further Epidemiological and Microbiological Studies Subgroup, North Thames, 1997. The molecular characterisation of *Cryptosporidium parvum* from two large suspected waterborne outbreaks. Commun Dis Public Health 1998;1:231-3.
11. Pieniazek NJ, Bornay-Llinares FJ, Slemenda SB, da Silva AJ, Moura INS, Arrowood M J, et al. New *Cryptosporidium* genotypes in HIV-infected persons. Emerg Infect Dis 1999;5:444-9.

12. Morgan UM, Weber R, Xiao L, Sulaiman I, Thompson RCA, Ndiritu W, et al. Molecular characterisation of *Cryptosporidium* isolates obtained from human immunodeficiency virus-infected individuals living in Switzerland, Kenya, and the United States. J Clin Microbiol 2000;38:1180-3.
13. Pedraza-Díaz S, Amar C, McLauchlin J. The identification and characterization of an unusual genotype of *Cryptosporidium* from human feces as *Cryptosporidium meleagridis*. FEMS Microbiol Lett 2000;189:189-94.
14. Arrowood MJ. Diagnosis. In: Fayer R, editor. *Cryptosporidium* and cryptosporidiosis. Boca Raton: CRC Press; 1997. p. 43-64.
15. Blewett DA. Quantitative techniques in *Cryptosporidium* research. In: Angus KW, Blewett DA, editors. Proceedings of the First International Workshop on Cryptosporidiosis. Edinburgh: The Animal Disease Research Association; 1989. p 85-95.
16. McLauchlin J, Casemore DP, Harrison TG, Gerson PJ, Samuel D, Taylor AG. The identification of *Cryptosporidium* oocysts by monoclonal antibody. Lancet 1987;i:51.
17. Patel S, Pedraza-Díaz S, McLauchlin J. The identification of *Cryptosporidium* species and *Cryptosporidium parvum* directly from whole feces by analysis of a multiplex PCR of the 18S rRNA gene and by PCR/RFLP of the *Cryptosporidium* outer wall protein (COWP) gene. Int J Parasitol 1999;29:1241-7.
18. Johnson DW, Pieniazek NJ, Griffin DW, Misener L, Rose JB. Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. Appl Environ Microbiol 1995;61:3849-55.
19. Bornay-Llinares FJ, da Silva AJ, Moura INS, Myjak P, Pietkiewicz H, Kruminis-Lozowska W, et al. Identification of *Cryptosporidium felis* in a cow by morphologic and molecular methods. Appl Environ Microbiol 1999;65:1455-8.
20. Bouchier I. *Cryptosporidium* in water supplies: Third report of the group of experts. London: Her Majesty's Stationery Office; 1998.
21. McLauchlin J, Casemore DP, Moran S, Patel S. The epidemiology of cryptosporidiosis: application of experimental sub-typing and antibody detection systems to the investigation of water-borne outbreaks. Folia Parasitol 1998;45:83-92.
22. Willocks L, Crampin A, Milne L, Seng C, Susman M, Gair R, et al. A large outbreak of cryptosporidiosis associated with a public water supply from a deep chalk borehole. Commun Dis Public Health 1998;1:239-43.
23. Surveillance of waterborne disease and water quality: January to June 1998. Commun Dis Public Health 1998;8:305-6.
24. Surveillance of waterborne disease and water quality. January to June 1999, and summary 1999. Commun Dis Public Health 1999;9:305-7.
25. Surveillance of waterborne disease and water quality: July to December 1999. Commun Dis Public Health 2000;10:65-7.
26. Sulaiman IM, Xiao L, Yang C, Escalante L, Moore A, Beard CB, et al. Differentiating human and animal isolates of *Cryptosporidium parvum*. Emerg Infect Dis 1998;4:681-5.
27. Ong CSL, Eisler DL, Goh SH, Tomblin J, Awad-el-Kariem FM, Beard CB, et al. Molecular epidemiology of cryptosporidiosis outbreaks and transmission in British Columbia, Canada. Am J Trop Med Hyg 1999;61:63-9.
28. Cacció S, Homan W, Camilli R, Traldo G, Kortbeek T, Pozio E. A microsatellite marker reveals population heterogeneity within human and animal genotypes of *Cryptosporidium parvum*. Parasitology 2000;120:237-44.
29. Quiroz ES, Bern C, MacArthur JR, Xiao L, Fletcher M, Arrowood MJ, et al. An outbreak of cryptosporidiosis linked to a food handler. J Infect Dis 2000;181:695-700.
30. MacKenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE, et al. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. N Engl J Med 1994;331:161-7.
31. Le Blancq SM, Khramtsov NV, Zamani F, Upton SJ, Wun TW. Ribosomal RNA gene organization in *Cryptosporidium parvum*. Mol Biochem Parasitol 1997;90:463-78.
32. Piper M, Bankier AT, Dear PH. A HAPPY map of *Cryptosporidium parvum.* Genome Res 1999;8:1299-1307.
33. Sulaiman IM, Xiao L, Lal AA. Evaluation of *Cryptosporidium* genotyping techniques. Appl Environ Microbiol 1999;65:4431-35.
34. McLauchlin J, Amar C, Pedraza-Díaz S, Nichols GL. Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal from livestock animals. J Clin Microbiol 2000;38:3984-90.
35. Okhuysen PC, Chappell CL, Crabb JH, Sterling CR, DuPont HL. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. J Infect Dis 1999;180:1275-81.
36. Carraway M, Tzipori S, Widmer G. A new restriction fragment length polymorphism from *Cryptosporidium parvum* identifies genetic heterogeneous parasite populations and genetic changes following transmission from bovine to human hosts. Infect Immun 1997;65:3958-60.
37. Casemore DP. Epidemiological aspects of human cryptosporidiosis. Epidemiol Infect 1990;104:1-28.

Preoperative Drug Dispensing as Predictor of Surgical Site Infection

Keith S. Kaye,* Kenneth Sands,* James G. Donahue,† K. Arnold Chan,† Paul Fishman,‡ and Richard Platt†
*Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts, USA; †Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA; ‡Group Health Cooperative of Puget Sound, Seattle, Washington, USA; and *†the Eastern Massachusetts CDC Prevention Epicenter, Boston, Massachusetts, USA

The system used by the National Nosocomial Infection Surveillance (NNIS) program to measure risk of surgical site infection uses a score of 3 on the American Society of Anesthesiologists (ASA)-physical status scale as a measure of underlying illness. The chronic disease score measures health status as a function of age, sex, and 29 chronic diseases, inferred from dispensing of prescription drugs. We studied the relationship between the chronic disease score and surgical site infection and whether the score can supplement the NNIS risk index. In a retrospective comparison of 191 patients with surgical site infection and 378 uninfected controls, the chronic disease score and ASA score were highly correlated. The chronic disease score improved prediction of infection by the NNIS risk index and augmented the ASA score for risk adjustment.

Approximately 325,000 surgical site infections occur each year in the United States, generating additional hospital costs in excess of $1 billion (1,2). Surgical site infection is also a major cause of increased hospital stay and death (3-6). Surgical site infection rates are an established measure of quality of clinical care (2,7), and reliable surveillance data are the foundation of effective infection control programs. However, to interpret surgical site infection surveillance rates, an effective risk adjustment system is needed. The National Nosocomial Infections Surveillance (NNIS) program uses a risk adjustment system for surgical site infection that includes three equally weighted variables: wound class, procedure duration, and the American Society of Anesthesiologists (ASA) score (8). The ASA score, a preoperative rating assigned to each patient, is a measure of the patient's general health status and coexisting conditions (9). Scores range from 1, representing a healthy person, to 5, representing a patient not expected to survive longer than 24 hours. The NNIS risk index assigns one point to patients with an ASA score ≥3. The ASA score is the only marker of coexisting conditions in the NNIS risk index.

Although the ASA score predicts surgical site infection, length of hospital stay, and risk for death (9-11), it is limited as a risk adjustment measure because of its subjectivity and poor inter-rater reliability (12-14). In a study in which 304 anesthesiologists assigned ASA scores to 10 hypothetical patients, the mean number of patients rated identically by the expert panel was 5.9 (13). The range of ASA scores is limited to five potential values; furthermore, there is often limited variation among patients undergoing similar procedures. Finally, the ASA score is not always available or easily accessible. It is typically not assigned for outpatient surgical procedures or procedures not attended by an anesthesiologist. An alternative rating system for coexisting diseases that does not have these limitations is needed.

A supplemental or alternative measure of a patient's underlying risk for surgical site infection is the chronic disease score, a measure

Address for correspondence: Keith S. Kaye, Duke University Medical Center, Box 3152, Durham, NC 27710; Fax: 919-684-3137; e-mail: Kaye0001@mc.duke.edu.

that predicts death, hospitalization, and use of health resources (15-17). For an individual patient, the chronic disease score is derived from the patient's age, sex, and presence or absence of each of 29 chronic diseases, which are inferred from ambulatory pharmacy dispensing records for the preceding 6 or 12 months.

We studied the relationships between surgical site infection and the chronic disease score and ASA score and evaluated whether the chronic disease score might improve or augment the NNIS risk index for surgical site infection.

Methods

Study Design

This nested case-control study involved cases of surgical site infection confirmed within 30 days after surgery, as well as individually matched controls. The study population was drawn from two patient cohorts (18,19) for which postoperative infection status and risk factors had been rigorously established. Data sources and methods of identifying surgical site infections have been described (18,19). We identified all adult members of the staff model component of Harvard Pilgrim Health Care (a mixed-model health maintenance organization) who underwent nonobstetric surgeries at Brigham and Women's Hospital from February 14, 1992, through March 6, 1993, and from May 19, 1997, through October 29, 1998. More than one surgery could be included for the same patient. Surgeries were excluded from analysis if they were performed on the same anatomic site within 3 months of prior surgery, on different sites but <1 month apart, or with infection as the indication for surgery. Patients were continuous members of the health-care plan for the 6 months before surgery and had prescription drug benefits. The NNIS classification system was used to categorize procedures on the basis of the International Classification of Diseases, 9th Edition (ICD-9) code (20). Surgical site infections occurring in the hospital or after discharge were identified by screening automated claims and electronic medical record data (18,19) and were confirmed by chart review according to NNIS criteria.

Controls were selected from patients who did not have a surgical site infection. Two controls were matched to each case from procedures performed within the following 6 weeks. In selecting controls, the greatest weight was given to matching procedure type (ICD-9 code, NNIS procedure, or NNIS procedure group), then to sex, age, and duration of procedure. The chronic disease score was calculated on the basis of prescriptions dispensed for the 6 months before surgery, available from an automated pharmacy record that captures essentially all pharmacy dispensing for health-plan members (17). Chronic disease scores have been reported on the basis of 6 or 12 months of pharmacy dispensing data. For this study, we used data from the 6 months before each patient's surgery, to minimize the number of exclusions caused by incomplete data. We used weights for disease classes derived for 12 months of data, because these provided a more current assessment of the importance of disease classes and because our emphasis was on the relative ranking of infected and uninfected patients, rather than on absolute risk prediction. We also computed the admission chronic disease score (a variant of the chronic disease score based solely on hospital pharmacy dispensing activity on the day of admission) by using an automated hospital database that captures all medications dispensed to hospitalized patients.

The ASA score, type of anesthesia administered, and emergency nature of the operative procedure were obtained by chart review. A procedure was considered an emergency if it was coded as such by the surgeon on the postoperative surgical summary sheets. Age, sex, procedure type, procedure duration, and wound class were obtained from automated databases.

Data Analysis

The chronic disease score was studied as a continuous, ordinal, and dichotomous predictor. As an ordinal predictor, the score was divided into quartiles. All quartiles were entered into a conditional logistic regression model. The effects were plotted to determine if they had increased in a linear fashion.

Choosing a breakpoint to create a dichotomous chronic disease score variable was problematic because of the overfitting that would be introduced if the breakpoint were based on the result of a single conditional logistic regression model. Therefore, we generated 500 bootstrap samples, each the same size as the entire dataset, and selected by resampling from our entire dataset. Then, using conditional logistic regression to select among 50 candidate breakpoints

after the data were controlled for age, sex, duration of surgery, and emergency surgery, we arrived at the best chronic disease score for each of the samples. A forward selection process was used to build the models; this process selected the chronic disease score, by producing 500 breakpoint values, of which the median value was chosen (21,22). In addition, the distribution of the 500 values was used to assess the stability and robustness of the final breakpoint. To determine a breakpoint for the admission chronic disease score, we followed the same procedure, except logistic regression was used rather than conditional logistic regression.

The ASA score was evaluated as both a 5-level ordinal variable, with values of 1,2,3,4, and 5, and as a dichotomous variable (ASA ≥ 3 and ASA <3, corresponding to the NNIS scoring system). The unadjusted relationship between chronic disease score and surgical site infection was analyzed by paired t test. All other analyses of the relationships between the chronic disease score and surgical site infection and between ASA and surgical site infection were performed by using conditional logistic regression. The unadjusted relationships between the admission chronic disease score and surgical site infection were analyzed with the Wilcoxon rank sum test and Fisher's exact test, because the cases of infection and their controls were no longer paired. Missing ASA scores (they were typically not available for ambulatory surgery procedures) were coded as dummy variables. Univariate relationships between other dichotomous variables and surgical site infection were analyzed with Cochran-Mantel-Haenszel summary statistics (for matched data) or Fisher's exact test (for unmatched data).

Relationships between continuous and ordinal variables were analyzed by conditional logistic regression for matched data and by Student's *t* test, Wilcoxon rank sum test, or logistic regression for unmatched data. The linearity assumption for continuous and ordinal predictors was examined in the same way as for the continuous chronic disease score. The relationships between the chronic disease score, the admission chronic disease score, and the ASA score were analyzed by Spearman correlation. For matched data, multivariate analyses assessing surgical site infection as the outcome were performed by conditional logistic regression. The following variables were included in the matched multivariate models: the chronic disease score, ASA score, type of anesthesia, emergency procedure vs. regular procedure, procedure duration, and wound class. The ability of these models to discriminate between infected and uninfected patients was compared by chi-square test to analyze differences in -2 log likelihood values.

Interaction terms between the chronic disease score and all other variables in the final model were evaluated and retained if they were statistically significant ($p<0.05$). For the unmatched analysis involving the admission chronic disease score, logistic regression was used. The following variables were included in the multivariate models: the admission chronic disease score, ASA score, sex, age, type of anesthesia, emergency procedure vs. regular procedure, procedure duration, and wound class. These regression models were analyzed for overfitting by the bootstrap method (1,000 bootstrap samples chosen as described).

An NNIS risk index score was calculated for each patient by assigning one point each for a contaminated wound, an ASA score ≥ 3, and surgical procedures lasting longer than the NNIS-derived 75th percentile for the duration of the procedure (23). Patients without an assigned ASA score were assumed to have a score <3, because more than two thirds of these surgeries were performed as outpatient procedures.

Two variant scores were also computed; in one of these, a chronic disease score $\geq 5{,}000$ was substituted for ASA score ≥ 3, and in the other, a point was added to the NNIS score for a chronic disease score $\geq 5{,}000$. The ability of these three scores to discriminate between infected and uninfected patients was compared by using unconditional logistic regression and the chi-square test to analyze differences in -2 log likelihood values. Unconditional regression was used to reduce the impact of our original selection process for uninfected controls. That process was influenced by procedure type and duration, both of which are part of the NNIS risk score. Analyses were performed with SAS software (SAS Institute, Cary, NC), system for Windows, v6.12).

Results

The source population for cases and controls was 9,037 patients who underwent 10,457 operative procedures. One hundred ninety-six confirmed surgical site infections were identified (infection rate 2.1%), and 392 matched controls

were selected. Drug dispensing data for the full 6 months before surgery were not available for 15 patients (5 cases and 10 controls), who were therefore excluded. If a case was excluded, its matched controls were also excluded (an additional 10 patients were excluded by this criterion).

The final study group included 191 cases and 372 controls (Table 1). The groups were comparable with regard to age (mean 51 years) and sex. General anesthesia (as opposed to other types of anesthesia) was associated with surgical site infection (Table 1). Although procedure duration was included in the multivariate matching process, longer duration was associated with surgical site infection. Procedures classified as emergency had borderline association. Other recognized risk factors, such as procedure type and NNIS wound class, were not significantly associated with infection because of the matching procedure that was used to select uninfected controls. ASA score was associated with infection, but chronic disease score was marginally associated.

The relationships were calculated between surgical site infection rates and ASA scores for the 80% of patients for whom scores had been assigned and between surgical site infection rates and the chronic disease scores (Figure 1). Patients with missing ASA scores were included in the chronic disease score groupings. The risk for surgical site infection increased with ASA group scores. In this group of patients (selected so that one third had a surgical site infection) 27.8% of those with an ASA score of 1, representing 25% of the study population, had surgical site infections, while 61.5% of those with scores of 4, representing 3% of the study population, had infections. No patients had scores of 5.

For the chronic disease score groups, the proportion with surgical site infections also

Table 1. Characteristics of the study population

Variable	Cases (n=191)	Controls (n=372)	p value	Odds ratio (95% CI)
Mean age (yrs)	51.2	51.5	NA	NA
Male sex (%)	91 (47.6)	182 (48.9)	NA	NA
NNIS wound class, contaminated or infected (%)	8 (4.2)	13 (3.5)	NA	NA
Surgical speciality (%)			NA	NA
General	54 (28.3)	107 (28.8)		
Cardiac	41 (21.5)	78 (21.0)		
Orthopedic	31 (16.2)	63 (16.9)		
Plastic	17 (8.9)	30 (8.1)		
Urologic	14 (7.3)	27 (7.3)		
Vascular	10 (5.2)	19 (5.1)		
Gynecologic	5 (2.6)	10 (2.7)		
Neurosurgical	4 (2.1)	8 (2.2)		
Other	15 (7.9)	30 (7.8)		
General anesthesia (%)	132 (69.1)	222 (59.7)	0.004	2.19 (1.29,3.72)
Duration of surgery, minutes (IQR)	105 (55-211)	83 (40-154)	<0.001[a]	1.75 (1.34,2,29)[a]
Emergent procedure (%)	11 (5.8)	10 (2.7)	0.07	2.21 (0.94,5.21)
ASA score (%)			0.002[b]	2.0 (1.4, 2.9)[b]
Missing	30 (15.7)	80 (21.5)		
1	32 (16.8)	83 (22.3)		
2	62 (32.5)	130 (34.9)		
3	59 (30.9)	74 (19.9)		
4	8 (4.2)	5 (1.4)		
5	0	0		
Chronic disease score				
Median (IQR)	2,219 (1,101-5,673)	1,641 (809-3,588)	0.09	NA

[a]Values are for duration as a 6-level ordinal variable. Risk is per-unit increase in duration category.
[b]ASA as a four-level ordinal variable, excluding missing values. Risk is per-unit increase in ASA category, missing values excluded.
OR = odds ratio.
ASA = American Society of Anesthesiologists physical status scale; IQR = Interquartile range.

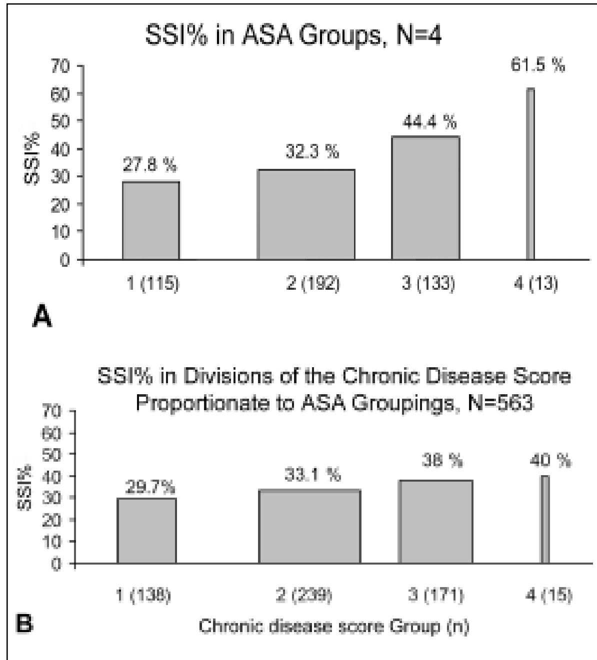


Figure 1 (A and B). Risk for surgical site infection in groups proportionate to ASA groupings for both the American Society of Anesthesiologists (ASA)-physical status score (A) and chronic disease score (B). The width of each bar is proportional to the sample size in that particular group. The percentage above each bar represents the proportion of persons in the group with infection.

increased with the chronic disease group scores. The group with the lowest 25% of the chronic disease scores had a surgical site infection proportion of 29.7%, while those with the highest 3% of scores had a surgical site infection proportion of 40%. The ASA score and the chronic disease score were strongly correlated (r=0.58, p<0.001). One major breakpoint in surgical site infection rates occurred between the lower 80% the upper 20% of the chronic disease score values, as determined by conditional logistic regression. Therefore, the chronic disease score was analyzed as a dichotomous variable, using a breakpoint of chronic disease scores ≥5,000 and <5000.

Relationships were calculated between surgical site infection and chronic disease scores >5,000 and between surgical site infection and ASA scores (Table 2a). In the unadjusted analysis (but with data controlled for the original matching variables of age, sex, and procedure type), both the chronic disease score ≥5,000 and ASA ≥3 were strong predictors of surgical site infection (for chronic disease score, odds ratio [OR] 2.6, 95% confidence interval [CI] 1.6-4.2, p<0.001; for ASA, OR 3.1, 95% CI 1.7-5.5, p<0.001). When added to a multivariate model

Table 2.

a. Relationships between chronic disease score, ASA score, and surgical site infection

Variable	Cases (n-191)	Controls (n=372)	Unadjusted[a] p value	Unadjusted[a] OR (95% CI)	Adjusted[b] p value	Adjusted[b] OR (95% CI)
Chronic disease score ≥5,000 (%)	55 (28.8)	60 (16.1)	<0.001	2.6 (1.6-4.2)	0.001	2.6 (1.5-4.7)
ASA ≥3 (%)	67(35.1)[c]	79 (21.2)[d]	<0.001	3.1 (1.7-5.5)	0.03	2.0 (1.1-3.7)

[a]Unadjusted values are controlled for age, sex, and procedure duration because of the matching process used to select uninfected subjects.
[b]Adjusted for chronic disease score ≥5,000, ASA ≥3, type of anesthesia, emergent nature of the procedure, procedure duration (ordinal), and wound class.
[c]Thirty (15.7%) cases had missing ASA scores.
[d]Eighty-two (21.7%) controls had missing ASA scores.
ASA = American Society of Anesthesiologists; OR = odds ratio; CI = confidence intervals

b. Relationships between admission chronic disease score, chronic disease score, ASA score and surgical site infection

Variable	Cases (n=51)	Controls (n=67)	Unadjusted p value	Unadjusted OR (95% CI)	Adjusted[a] p value	Adjusted[a] OR (95% CI)
Admission chronic disease score ≥4,500 (%)	18 (35)	5 (7)	<0.001	6.8 (2.3-19.9)	0.003	6.2 (1.9-20.4)
Chronic disease score ≥5,000 (%)	22 (43)	17 (25)	0.05	2.2 (1.0-4.9)	0.32	1.6 (0.6-3.9)
ASA ≥3 (%)	39 (83)[b]	36 (58)[c]	0.007	3.5 (1.4-8.8)	0.03	3.4 (1.1-10.2)

[a]Adjusted for sex, age, type of anesthesia, emergency nature of the procedure, procedure duration (ordinal) and wound class.
[b]Four cases had missing ASA scores.
[c]Five controls had missing ASA scores.

that already included the ASA score and other factors associated with surgical site infection, the chronic disease score improved the explanatory value of the model ($p<0.001$). In this model, both ASA and the chronic disease score remained significant predictors of surgical site infection (chronic disease score ≥5000, OR 2.6, 95% CI 1.5-4.7, p=0.001; ASA ≥3, OR 2.0, 95% CI 1.1-3.7, p=0.03).

A chronic disease score based solely on admission medications (admission chronic disease score) was also studied (Table 2b). From 191 total cases of surgical site infection and 372 uninfected controls, cases with admission and surgery on the same day were excluded, leaving 51 cases and 67 controls. The median admission chronic disease score for cases was 2,218 (interquartile range [IQR] 1,285-4,818) and for controls was 1,285 (IQR 1,209-2,729) (p=0.008, Wilcoxon rank sum test). The admission chronic disease score was correlated with the 6-month chronic disease score ($p<0.001$, r=0.45) and with the ASA score (p=0.02, r=0.22). The admission chronic disease score was analyzed as a dichotomous variable, using a breakpoint of chronic disease score ≥4,500 and chronic disease score <4,500. Admission chronic disease score ≥4,500, chronic disease score ≥5,000 and ASA ≥3 were all associated with surgical site infection; the association was strongest for the admission chronic disease score (for admission chronic disease score, OR 6.8, $p<0.001$; for the 6-month chronic disease score, OR 2.2, p=0.05; for ASA, OR 3.5, p=0.007).

After the data were controlled for anesthesia type, emergency nature of surgery, sex, age, procedure duration, and wound class by logistic regression, the model that included the admission chronic disease score had better predictive value for surgical site infection than the model containing the chronic disease score based on 6 months of preoperative medications ($p<0.01$) and the model that included the ASA score ($p<0.03$). In these multivariate models, the admission chronic disease score was a stronger predictor of surgical site infection than the chronic disease score based on 6 months of preoperative medications and the ASA score (for the admission chronic disease score, OR 6.2, p=0.003; for the 6-month chronic disease score, OR 1.6, p=0.32; for ASA score, OR 3.4, p=0.03); the ASA score did not improve the model already containing the admission chronic disease score (p=0.1).

In this population, the NNIS risk index was correlated with infection status (Figure 2A). As the risk index score increased, the proportion of infected patients increased (27% for a risk score of 0, 100% for a risk score of 3). Both the risk index that substituted the chronic disease score for the ASA score (Figure 2B) and the risk index that added the chronic disease score to the conventional NNIS score (Figure 2C) increased progressively in the proportion infected in each risk class. After the data were controlled for type

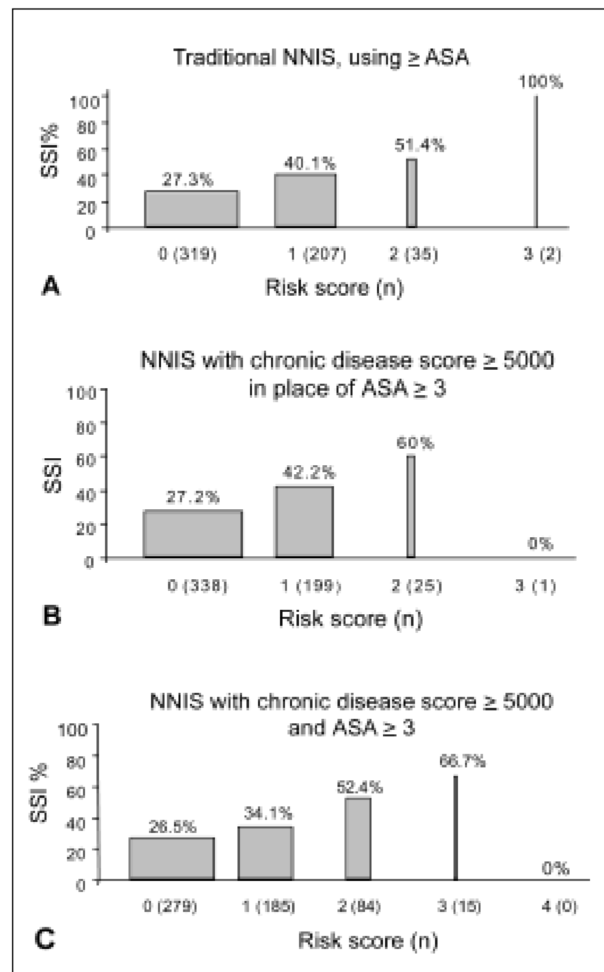


Figure 2 (A, B, and C). Risk of surgical site infection in different risk index categories. The width of each bar is proportional to the sample size in that particular group; A) shows the traditional National Nosocomial Infection Surveillance (NNIS) risk index categories; B) shows a modified NNIS risk index with chronic disease score ≥5,000 substituted for ASA ≥3; C) shows a modified NNIS risk index, incorporating both chronic disease score ≥5,000 and the traditional NNIS risk index categories. In each group, the percentage of patients with infections is shown.

of anesthesia used and emergency nature of the surgery, the risk index that included a chronic disease score ≥5,000 in place of ASA ≥3 (Figure 2B) was a stronger predictor of surgical site infection than the traditional NNIS score (Figure 2A) ($p<0.05$). The risk index that included both a chronic disease score ≥5,000 and an ASA ≥3 (Figure 2C) was a better predictor of surgical site infection than both the traditional risk score ($p<0.001$) and the score that substituted the chronic disease score for an ASA ≥3 ($p<0.01$).

Conclusions

The chronic disease score, like the ASA score when other known risk factors for infection were taken into consideration, was a strong predictor of postoperative surgical site infection. The strength of the association between the ASA score and the chronic disease score was impressive since the two scores are derived by completely different methods. The chronic disease score performed comparably with the ASA score as a component of the NNIS risk index, and an NNIS-like index based on both the chronic disease and ASA scores performed better than the conventional NNIS index. In our limited sample of patients who were admitted to the hospital before the day of surgery, a chronic disease score based solely on medications dispensed on the day of admission was a stronger predictor of surgical site infection than either the ASA or 6-month chronic disease scores. Thus, either the 6-month or admission chronic disease scores might be considered as an alternative to the ASA score and may provide better risk stratification.

Compared with the ASA score, the chronic disease score has several potential advantages as a risk-adjuster for surgical site infection. It is objective and, since it can be calculated from the administrative databases of health insurance organizations, sometimes more readily available than the ASA score. Pharmacy databases are often the most complete and accurate ones maintained by health plans, and the information in these systems is usually easy to interpret. In addition, pharmacy data can often be obtained from third parties, such as pharmacy benefit managers, or directly from the pharmacies where prescriptions were filled.

Since the chronic disease score can be derived from electronic pharmacy data, it lends itself to use as a risk-adjuster in automated systems for monitoring surgical site infection, particularly in patients discharged from the hospital who do not return to the hospital for care. Such systems would be applicable not only for the surveillance and risk adjustment of surgical site infection rates for individual hospitals, but also for larger populations such as members of a health maintenance organization or other health insurance group. The chronic disease score based solely on admission medications might be attractive for use in hospital-based surveillance systems if the results in this study are reproducible in larger patient populations.

This study had some limitations. Since age is a component of the chronic disease score and cases were matched to controls by age, the observed association between the chronic disease score and surgical site infection may be underestimated. In addition, because components of the NNIS risk index were used to match uninfected controls to cases, our results may underestimate the associations between surgical site infection and the traditional NNIS risk index, the risk index using the chronic disease score in place of an ASA score, and the index incorporating both the chronic disease and ASA scores. Additionally, all the procedures were performed at a single hospital, increasing the likelihood that ASA scores were assigned more consistently than in the population at large. Such an effect would have overestimated the utility of the ASA score. Restriction to a single institution may also affect the generalizability of these results. Although we do not have a ready explanation for ways in which the chronic disease score could have a special relationship to infection in this population, our findings should be tested in other settings. Our relatively limited sample size precludes investigation of whether the chronic disease score performed differently for different types of procedures or specific groups of patients. Validation of our model in a larger population with a more diverse set of procedure types would be worthwhile. Finally, the chronic disease score can only be assessed readily for patients whose pharmacy dispensing data are available. This information exists for most patients through pharmacy benefit systems, but few hospitals have ready access to this information.

Aspects of the association between chronic disease score and surgical site infection that warrant additional exploration include assessing

the value of the chronic disease score, either alone or in addition to the ASA score for specific procedure types, and developing new weights for the chronic disease categories to improve predictive value. Evaluation of a chronic disease score based on admission medications using a larger sample size than the one used in this study and including patients from multiple institutions would be of interest.

In summary, the chronic disease score provides a useful risk-adjuster for surgical site infection. It is objective and can often be obtained from automated information available from standard health insurance claims data. It may be available when the ASA score is not, so it could be used either in place of the ASA score or in addition to it. Further investigation of the chronic disease score and its association with surgical site infection is warranted.

Acknowledgments

We thank E. John Orav for his expert statistical guidance.

This study was presented in part at the Society for Healthcare Epidemiology of America annual meeting, San Francisco, California, April 1999, and at the 4th Decennial International Conference on Nosocomial and Health Care-Associated Infections, Atlanta, Georgia, March 2000.

This work was supported by the Harvard Pilgrim Health Care Foundation and Cooperative Agreement UR8/CCU115079 from the Centers for Disease Control and Prevention.

Dr. Kaye is the associate hospital epidemologist and an infectious disease attending physician at Duke University Medical Center. His professional interests include hospital epidemiology and efficient animicrobial drug utilization. His academic interests focus on the study of outcomes and risk factors associated with multidrug-resistant bacterial pathogens and surgical site infections.

References

1. Mayhall CG. Surgical infections, including burns. In: Wenzel RP, editor. Prevention and control of nosocomial infections. 2nd ed. Baltimore: Williams & Wilkins; 1993:p. 614-64.
2. Wenzel RP. Nosocomial infections, diagnosis-related groups, and study on the efficacy of nosocomial infection control: economic implications for hospitals under the prospective payment system. Am J Med 1985;78:3-7.
3. Cruse PJ, Foord R. The epidemiology of wound infection: a 10-year prospective study of 62,939 wounds. Surg Clin North Am 1980;60:27-40.
4. Cruse P. Wound infection surveillance. Rev Infect Dis 1981;3:734-7.
5. Boyce JM, Potter-Bynoe G, Dziobek L. Hospital reimbursement patterns among patients with surgical wound infections following open heart surgery. Infect Control Hosp Epidemiol 1990;11:89-93.
6. Poulsen KB, Bremmelgaard A, Sorensen AI, Raahave D, Petersen JV. Estimated costs of postoperative wound infections: a case-control study of marginal hospital and social security costs. Epidemiol Infect 1994;113:283-95.
7. Haley RW, Culver DH, White JW, Morgan WM, Emori TG, Munn VP, et al. The efficacy of infection surveillance and control programs in preventing nosocomial infections in US hospitals. Am J Epidemiol 1985;121:182-205.
8. Centers for Disease Control and Prevention. Draft guidelines for the prevention of surgical site infection, 1998. Federal Registister 1998;63:33168-92.
9. Cullen DJ, Apolone G, Greenfield S, Guadagnoli E, Cleary P. ASA Physical Status and age predict morbidity after three surgical procedures [comments]. Ann Surg 1994;220:3-9.
10. Vacanti CJ, VanHouten RJ, Hill RC. A statistical analysis of the relationship of physical status to postoperative mortality in 68,388 cases. Anesth Analg 1970;49:564-6.
11. Garibaldi RA, Cushing D, Lerer T. Risk factors for postoperative infection. Am J Med 1991;91:158S-163S.
12. Haynes SR, Lawler PG. An assessment of the consistency of ASA physical status classification allocation [see comments]. Anaesthesia 1995;50:195-9.
13. Owens WD, Felts JA, Spitznagel EL Jr. ASA physical status classifications: a study of consistency of ratings. Anesthesiology 1978;49:239-43.
14. Salemi C, Anderson D, Flores D. American Society of Anesthesiology scoring discrepancies affecting the National Nosocomial Infection Surveillance System: surgical-site-infection risk index rates. Infect Control Hosp Epidemiol 1997;18:246-7.
15. Von Korff M, Wagner EH, Saunders K. A chronic disease score from automated pharmacy data. J Clin Epidemiol 1992;45:197-203.
16. Fishman P, Goodman M, Hornbrook M, Meenan R, Bachman D, O'Keefe-Rosetti M. Risk adjustment using automated using pharmacy data: a global chronic disease score. 2nd International Health Economic Conference, June 8, 1999, Rotterdam, the Netherlands, 1999.
17. Clark DO, Von Korff M, Saunders K, Baluch WM, Simon GE. A chronic disease score with empirically derived weights. Med Care 1995;33:783-95.
18. Sands K, Vineyard G, Platt R. Surgical site infections occurring after hospital discharge. J Infect Dis 1996;173:963-70.

19. Sands K, Vineyard G, Livingston J, Christiansen C, Platt R. Efficient identification of postdischarge surgical site infections: use of automated pharmacy dispensing information, administrative data, and medical record information. J Infect Dis 1999;179:434-41.
20. Emori TG, Culver DH, Horan TC, Jarvis WR, White JW, Olson DR, et al. National nosocomial infections surveillance system (NNIS): description of surveillance methods. Am J Infect Control 1991;19:19-35.
21. Hosmer DW, Lemeshow S. Applied logistic regression. New York: John Wiley & Sons; 1989.
22. Efron B, Tibshirani RJ. An introduction to the bootstrap. New York: Chapman & Hall; 1993.
23. Culver DH, Horan TC, Gaynes RP, Martone WJ, Jarvis WR, Emori TG, et al. Surgical wound infection rates by wound class, operative procedure, and patient risk index. National Nosocomial Infections Surveillance System. Am J Med 1991;91:152S-7S.

Lack of Evidence of Endogenous Avian Leukosis Virus and Endogenous Avian Retrovirus Transmission to Measles, Mumps, and Rubella Vaccine Recipients

Althaf I. Hussain,* Vedapuri Shanmugam,* William M. Switzer,* Shirley X. Tsang,* Aly Fadly,† Donald Thea,‡ Rita Helfand,* William J. Bellini,* Thomas M. Folks,* and Walid Heneine*
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Avian Disease and Oncology Laboratory, U.S. Department of Agriculture, East Lansing, Michigan, USA; ‡Harvard Institute for International Development, Cambridge, Massachusetts, USA

The identification of endogenous avian leukosis virus (ALV) and endogenous avian retrovirus (EAV) in chick cell-derived measles and mumps vaccines in current use has raised concern about transmission of these retroviruses to vaccine recipients. We used serologic and molecular methods to analyze specimens from 206 recipients of measles, mumps, and rubella (MMR) vaccine for evidence of infection with ALV and EAV. A Western blot assay for detecting antibodies to endogenous ALV was developed and validated. All serum samples were negative for antibodies to endogenous ALV by Western blot analysis. Peripheral blood lymphocyte samples from 100 vaccinees were further tested by polymerase chain reaction for both ALV and EAV proviral sequences; all were negative. Matching serum samples were tested by reverse transcriptase polymerase chain reaction for ALV and EAV RNA, and all 100 samples were negative, providing no evidence of viremia. These findings do not indicate the presence of either ALV or EAV infection in MMR vaccine recipients and provide support for current immunization policies.

Vaccines effectively reduce and prevent death and disease from many viral infections. However, vaccine production occasionally has been complicated by inadvertent contamination with adventitious agents that may have originated from cell substrates used to propagate vaccine strains. Examples of such contamination include simian virus in early polio vaccines grown on monkey kidney cells and avian leukosis virus (ALV) in yellow fever vaccines propagated in chick embryos (1). Hepatitis B virus has also been identified in yellow fever vaccines produced by using pooled human serum as a stabilizing agent (2). Exposure of vaccine recipients to contaminated vaccines has been associated with effects ranging from benign to demonstrable transmission of infection, with or without subsequent disease (2,3).

Address for correspondence: Walid Heneine, Centers for Disease Control and Prevention, 1600 Clifton Road, Mail Stop G19, Atlanta, GA 30333; fax: 404-639-1174; e-mail: whm2@cdc.gov.

Reverse transcriptase (RT) activity, an indicator of retroviruses, has recently been detected by sensitive polymerase chain reaction (PCR)-based RT assays in currently used vaccines produced in chick embryo fibroblasts or embryonated eggs (4-7). The RT-positive vaccines include measles, mumps, and yellow fever vaccines produced by several manufacturers in Europe and the United States (4,5). RT activity was detected in the vaccines despite strict manufacturing practices requiring that chick embryos and embryo fibroblasts be derived from closed, specific-pathogen-free chicken flocks. Such chickens are screened for known pathogens, including two exogenous avian retroviruses: reticuloendotheliosis virus and ALV (8).

The origin of RT activity in measles vaccines was examined in two recent studies. RT activity

in a vaccine manufactured in Europe was associated with particles containing endogenous avian virus (EAV) RNA (6). In the second study, we examined measles vaccines from a U.S. manufacturer and found evidence of both EAV and endogenous ALV (7): we detected particle-associated ALV and EAV-0 RNA sequences in both vaccine and chick embryo fibroblast supernatants and demonstrated neutralization of RT activity in vaccines by anti-ALV RT antibodies. In addition, we observed ALV-like particles by electron microscopy in culture supernatants from chick embryo fibroblasts that had not been inoculated with vaccine viruses (7).

At least six subgroups of ALV (A-E and J) have been identified in chickens on the basis of differences in envelope sequences (9). Only subgroup E viruses are expressed from endogenous sequences that are part of the chicken germ line; all the other subgroups are exogenous. The endogenous ALV sequences are usually referred to as endogenous viral (*ev*) loci. At least 30 *ev* loci have been characterized in various chicken strains (10). Although endogenous ALVs are not known to be pathogenic for chickens, related species of fowl are susceptible to infection with endogenous ALV (11). Disease associations in these cross-species infections have not been fully investigated (11). Exogenous-type ALVs have been shown to cause several neoplastic diseases in infected chickens (9).

Less is known about EAV, which has elements distinct from but closely related to those of the ALV family of endogenous retroviruses. EAV are also present in line-0 chickens (*ev*-negative), which have been bred to have no *ev* proviruses (12). EAV elements exist in at least 50 copies per chicken genome (13).

The observed association of the RT activity of these vaccines with endogenous retroviral particles rather than exogenous retroviruses is consistent with vaccine manufacturing regulations that require exogenous ALV and reticuloendotheliosis virus infections to be eliminated from source chickens. Endogenous retroviral particles are not addressed by current manufacturing guidelines because these particles had not been associated with chick cell-derived vaccines.

The finding of RT activity in all measles vaccine lots from different manufacturers tested suggests that this occurrence is not sporadic and that vaccine recipients may be universally exposed to these retroviral particles (4,5,7,14). Surveillance for infection with ALV/EAV in vaccine recipients is important for evaluating the safety of these vaccines. This surveillance, which was recommended by the World Health Organization in a consultation meeting on RT activity in chicken cell-derived vaccines, is needed for policy decisions regarding the global use of these vaccines (15). We recently reported negative PCR results for ALV and EAV sequences in peripheral blood lymphocytes from 33 measles, mumps, and rubella (MMR) vaccine recipients (7). However, these preliminary results do not fully reflect risks for transmission of ALV and EAV because of the small number of samples analyzed and the lack of testing for antibodies and plasma viremia (7). We have expanded our surveillance for ALV and EAV infection in recipients of MMR vaccines and here report evidence that does not support infection with either ALV or EAV.

Materials and Methods

Study Population

The study population was 206 children identified from two cohorts. Samples for 113 of the children were identified from repository serum specimens of the New York City Perinatal HIV Transmission Collaborative Study and Perinatal AIDS Collaborative Transmission Study (PACTS). All 113 children had documented evidence of MMR vaccination; none were infected with HIV-1 (16). The remaining 93 children participated in a study of antibody responses to immunization with the U.S.-manufactured MMR vaccine (17). Of 206 specimens analyzed, 32 (15.5%) were collected 6 to 12 months and 158 (76.7%) 12 to 24 months after the first MMR vaccination. Sixteen (7.8%) samples were collected after the second MMR dose. Peripheral blood lymphocytes samples were available for 100 of 113 children from the PACTS study. All testing was done anonymously with regard to children's identity.

Endogenous ALV-based Western Blot Assay

The source of antigen for the Western blot assay was the Rous-associated virus type 0 (RAV-0), a prototype endogenous ALV highly related to the endogenous ALV particles found in MMR vaccine (9,10,18). RAV-0 was inoculated into $15B_1$ chick embryo fibroblast cells. Infection with

RAV-0 was monitored by using the ALV antigen test kit (Flockchek, IDEXX, ME) that detects ALV p27 *gag* antigen. Antigen was prepared by lysing 10^6 cells with 100 µL lysis buffer, followed by 5 minutes each of boiling and sonication. The protein concentration of the lysate was determined with the Pierce protein kit (Rockford, IL).

Electrophoresis was done on 150 µg of either infected or uninfected whole-cell lysates in 10%-20% Tris-HCl gradient SDS-polyacrylamide gels (BioRad, CA) for 2 hours at 70V. Serum samples were diluted 1:50 in blocking buffer. Rabbit anti-avian myeloblastosis virus (AMV, an exogenous strain of ALV) p27 (SPAFAS, Preston, CT) antibody was used as positive control. Anti-rabbit, anti-chicken, and anti-human antibodies conjugated to horseradish peroxidase were used as secondary antibodies for rabbit, chicken, and human plasma samples at 1:6000, 1:3000, and 1:6000 dilutions, respectively. Control human IgG was used as an assay control for anti-human horseradish peroxidase-conjugated secondary antibody.

To validate the Western blot assay, sera from 27 chickens seropositive by virus neutralization assays for ALV (subgroup A) and 34 ALV-seronegative chickens were tested for seroreactivity to ALV antigens by Western blot. In addition, 10 serum samples from chickens infected with reticuloendotheliosis virus were used as specificity controls. Validation on human sera included testing samples from persons infected with human T-cell lymphotropic virus types I and II (HTLV-I and HTLV-II) and HIV types 1 and 2 to assess possible cross-reactivity between ALV and human retroviruses. HTLV- and HIV-negative sera from anonymous blood donors were also included in this validation. All chicken serum samples used for validation of the RAV-0 Western blot assay were also tested on blots containing control antigen from uninfected $15B_1$ chick embryo fibroblasts.

Proviral DNA PCR

Aliquots of lysates from 150,000 peripheral blood lymphocytes from MMR vaccine recipients were amplified by PCR for ALV *env* and EAV *env*-like sequences by using primers ALVENVF2/ALVENVR2 and EAVENVF10/EAVENVR10, respectively (7). All diagnostic primers used were derived from particle-associated viral sequences identified in the vaccine substrate used to prepare the MMR vaccine (7). Both assays are highly sensitive, with a detection threshold of one copy for EAV PCR assay and 1-10 copies for the ALV PCR assay (7). The PCR reaction conditions included 35 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. PCR products were detected by Southern blot hybridization to the ALV- and EAV-specific ^{32}P-labeled probes, ALVENVP1 and EAVENVP1, respectively (7).

Detection of ALV and EAV RNA in Vaccine Recipients

RNA was extracted from serum as described (19). The primers used for the RT reaction were ALVENVR2 and EAVENVR10 for ALV and EAV, respectively. The reaction was carried out at 37°C for 2 hours, followed by 95°C for 5 minutes. RNA extracted from RAV-0-infected $15B_1$ chick embryo fibroblast supernatants was used for positive controls. PCR was carried out as described (7). The ALV and EAV PCR products were detected by Southern blot hybridization with the ^{32}P-labeled ALVENVP1 and EAVENVP1 probes, respectively.

Results

Validation of Western Blot Assay and Criteria for Positivity

The presence of viral proteins was confirmed by the use of antisera raised against whole ALVs (anti-AMV and anti-RAV-0) and against anti-p27 gag protein from AMV (Table). These antisera detected *env* gp85 and gp37 as well as *gag* p27,

Table. Western blot antibody reactivity to the p27 gag protein of the endogenous avian leukosis virus (ALV) in vaccine recipients and other reference chicken and human sera

Sera tested	p27 Positive
Chicken sera (n = 61)	
ALV infected/antibody positive[a]	27/27
ALV uninfected/antibody negative[a]	0/34
REV infected	0/10
Human sera (n = 68)	
Blood donors	0/60
HIV-1/2 positive	0/4
HTLV-I/II positive	0/4
MMR vaccine recipients (n = 206)	
6-12 months[b]	0/32
12-30 months[b]	0/158
6-12 months[c]	0/16

[a]antibody reactivity to ALV determined by virus neutralization assays.
[b]samples collected after first MMR vaccination.
[c]samples collected after second MMR vaccination.
REV = reticuloendotheliosis virus; HIV-1/2 = human immunodeficiency virus type 1 or 2; HTLV-I/II = human T-cell lymphotropic virus type 1 or 2; MMR = measles, mumps and rubella

p19, p15, and p12 proteins (data not shown). All 27 ALV-infected and neutralization antibody-positive chicken sera reacted strongly to RAV-0 p27, while negative control sera from both uninfected chickens and reticuloendotheliosis virus-infected chickens showed no reactivity to p27 (Figure 1a). These data support the use of p27 reactivity as a marker for ALV seropositivity. Similarly, negative results were seen with samples from 60 human blood donors. No cross-reactivity was observed between RAV-0 p27 *gag* protein and antibodies against HIV-1, HIV-2, HTLV-I, and HTLV-II (data not shown).

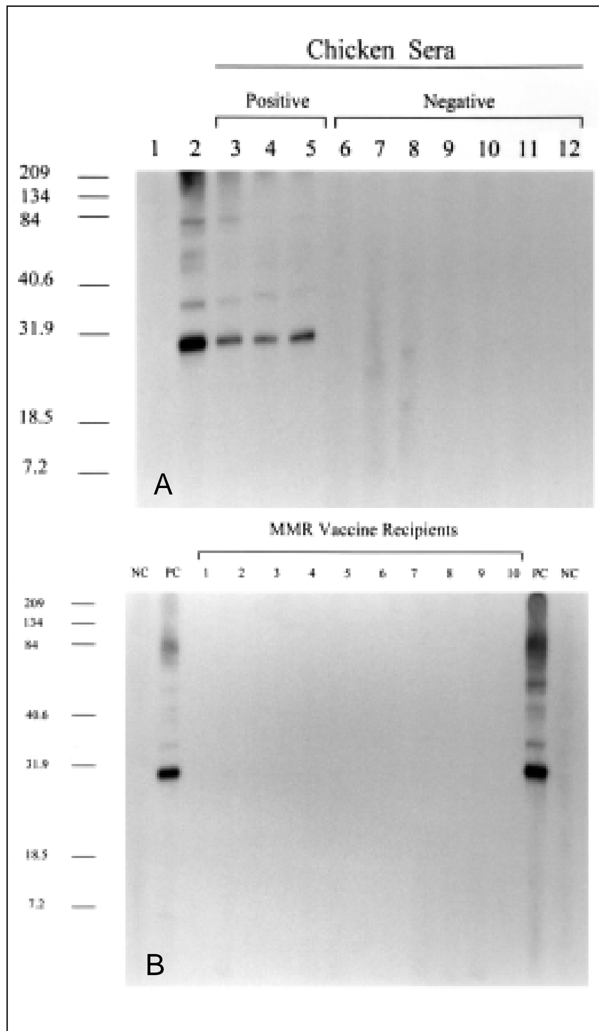


Figure 1. Western blot antibody reactivity of chicken sera to endogenous avian leukosis virus (ALV) (Rous-associated virus 0) antigen. a) Lane 1, negative control chicken serum; Lane 2, positive control anti-p27 ALV gag antiserum; lanes 3-5, sera from ALV antibody-positive chickens; lanes 6-12, sera from ALV-negative, antibody-negative chickens. b) NC, negative control human sera; PC, positive control: anti-p27 ALV gag antiserum; lanes 1-10, sera from measles mumps rubella vaccine recipients.

Lack of Evidence of Seroreactivity to ALV

Serum samples from all 206 MMR vaccine recipients were negative by Western blot (Table). These samples included those of the 16 children who had received two doses of MMR vaccine (Figure 1b). No seroreactivity to any viral proteins, including p27, was observed.

Lack of Evidence of ALV and EAV Sequences

All 100 peripheral blood lymphocyte samples were negative for both ALV and EAV DNA sequences by PCR analysis (Figure 2). Of the 100 samples from the PACTS cohort, 33 had been tested previously (7). Similarly, all sera from the 100 children tested negative for both ALV and EAV RNA by RT-PCR (Figure 3). These results indicate absence of both ALV and EAV viremia in these vaccine recipients (Table).

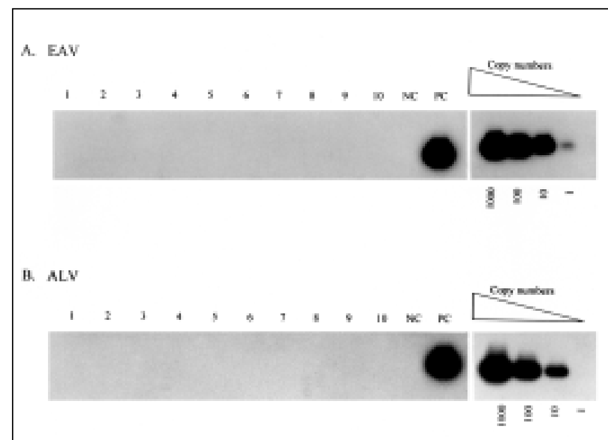


Figure 2. Representative results from polymerase chain reaction analysis of peripheral blood lymphocytes from measles, mumps and rubella (MMR) vaccine recipients for endogenous avian retrovirus (EAV) (A) and avian leukosis virus (ALV) (B) proviral DNA sequences. The detection threshold of known copy numbers of the target sequences is shown in the righthand panels. NC, negative control, uninfected human peripheral blood lymphocytes; PC, positive control, human peripheral blood lymphocytes spiked with 1,000 copies of target sequence; Lanes 1-10, peripheral blood lymphocytes from MMR vaccine recipients.

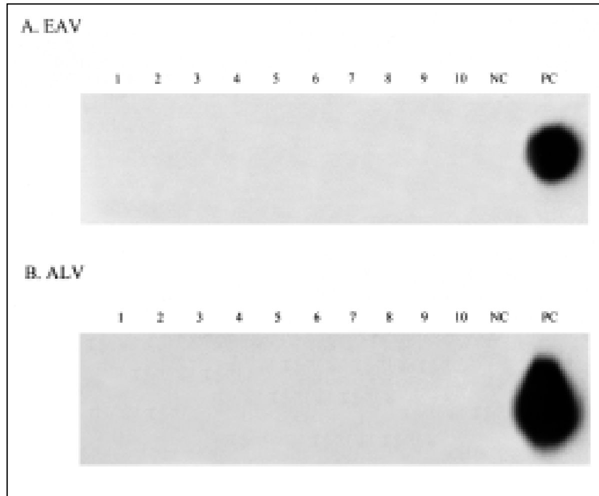


Figure 3. Reverse transcriptase-polymerase chain reaction analysis of sera from measles, mumps and rubella (MMR) vaccine recipients for the presence of avian leukosis virus (ALV) (A) and endogenous avian retrovirus (EAV) (B) RNA. Lanes 1-10, samples from MMR vaccine recipients. NC, negative control, uninfected human serum; PC, positive control, culture supernatant from Rous-associated virus 0 (RAV-0) infected $15B_1$ chick embryo fibroblasts.

Conclusions

Analysis of MMR vaccines from different manufacturers suggests that vaccine recipients may be universally exposed to endogenous chicken retroviral particles. We sought evidence of persistent ALV and EAV infection in a large number of MMR vaccine recipients, and we were unable to find any evidence of ALV or EAV sequences in peripheral blood lymphocytes, despite the use of highly sensitive PCR assays. Neither did we find evidence of ALV or EAV viremia, since all serum samples tested negative for ALV and EAV RNA by RT-PCR analysis; this finding is of interest because ALV viremia is commonly seen in chickens infected with ALV (18). All 206 serum samples tested by a validated Western blot assay were negative for ALV antibodies, indicating absence of antigenic exposure. These findings differ from those in persons infected with human retroviruses, who usually seroconvert 2 weeks to 6 months after exposure (20,21). The negative serologic data also suggest the low likelihood of nonviremic ALV infection in cells other than peripheral blood lymphocytes, which may not have been detected by PCR testing. Our results overall show no evidence of infection with either ALV or EAV in these vaccine recipients. The lack of transmission of ALV and EAV observed in 16 children who had two MMR vaccinations provides additional reassuring data.

Several factors, including a natural human resistance to infection with endogenous ALV, may explain the lack of transmission of these viruses to MMR vaccine recipients. However, few or no data are available on the ability of endogenous ALV to replicate in human cells. Resistance to endogenous ALV infection may, for instance, be attributed to the absence of a human cell-surface receptor for the virus as well as to other intracellular blocks for ALV replication. A tumor necrosis factor receptor-related protein, referred to as SEAR, has been recently identified as a receptor for endogenous ALV in turkey cells (22). Plasmid-encoded expression of SEAR in human 293 cells can confer susceptibility to infection by endogenous ALV, suggesting that human cells can support endogenous ALV replication if virus entry is achieved (22). Human serum can lyse ALV by complement activation (23); however, this protective mechanism has not been demonstrated for endogenous ALV and EAV particles.

The presence of defective ALV and EAV particles in vaccines may also explain the lack of transmission of these viruses to vaccine recipients. *ev* loci confer a variety of different phenotypes, including infectious or defective particles (9,10,18,24). However, it is not known whether the ALV particles in the vaccine are all defective. The proportion of defective to infectious ALV in different vaccine lots depends on the set of the *ev* loci in the chick embryo fibroblast substrate preparation used for each vaccine lot. Loci associated with noninfectious viruses (*ev*-1, *ev*-3, and *ev*-6) have been identified in a chick embryo fibroblast substrate of a U.S. vaccine manufacturer (7). However, the presence of many loci known to produce infectious ALV-E could not be determined (7).

EAV may represent the predominant retroviral particles in MMR vaccines (6,7). Therefore, our data, which indicate that exposure to EAV particles was not associated with EAV viremia or EAV-infected peripheral blood lymphocytes in vaccine recipients, are important. Confirmation of our molecular results by EAV-specific serologic testing may, however, be necessary. The lack of evidence of transmission of

EAV to vaccinees is likely due to the presence of defective particles. No infectious EAVs have yet been isolated, nor has a full-length intact EAV provirus been identified (25). However, our understanding of the EAV family is limited.

The presence of ALV in chick-cell-derived vaccines is not a new phenomenon; many instances of ALV contamination in yellow fever and measles vaccines have been documented (26,27). However, earlier vaccines had evidence of exogenous rather than endogenous type ALV (27). Available data also suggest lack of transmission of ALV to vaccine recipients (26,28,29). These studies examined 6 adults and 41 children with measles vaccination and 227 yellow fever vaccine recipients (26,27,29), and no evidence was found of neutralizing antibodies to an exogenous ALV. No increase in cancer rate has been found in a study of recipients of exogenous ALV-positive yellow fever vaccine (27).

Despite these reassuring data, the presence of avian retroviral particles in chick embryo fibroblast-derived vaccines raises questions about the suitability of primary chicken cell substrates for vaccine production and the advisability of a change to RT-negative substrates. Chick embryo fibroblasts originating from line 0 chickens could provide substrates that do not express ALV-E; however, such cells may still produce EAV particles (7,12). Obtaining an RT-negative substrate may require a substantial change from primary chicken cells to RT-negative cells from different species, such as immortalized or diploid mammalian cells. Since the cell substrate is critical to the attenuation of live vaccine viruses, any change in the cell substrate could have unpredictable effects on the safety and efficacy of the vaccine and should be approached cautiously.

In conclusion, we found no evidence of either endogenous ALV or EAV infection in recipients of U.S.-made MMR vaccines. Our data indicate that no change is warranted in current U.S. policies for the use of MMR vaccine.

Acknowledgments

We thank Alison Mawle, Harold Jaffe, and Rafael Harpaz for critical reviews of the manuscript.

This work was supported in part by The National Vaccine Program Office.

Dr. Hussain is a postdoctoral fellow at the Molecular Epidemiology and Zoonoses Section, HIV and Retrovirology Branch, CDC, with research interests in investigating retroviral zoonotic infections.

References

1. Parkman PD. Safety of biopharmaceuticals: a current perspective. Dev Biol Stand 1996;88:5-7.
2. Norman JE, Gilbert WB, Jay HH, Leonard BS. Mortality follow-up of the 1942 epidemic of hepatitis B in the U.S. Army. Hepatology 1993;18:790-7.
3. Fisher SG, Weber L, Carbone M. Cancer risk associated with simian virus 40 contaminated polio vaccine. Anticancer Res 1999;19:2173-80.
4. Boni J, Stadler J, Reigei F, Shupbach J. Detection of reverse transcriptase activity in live attenuated virus vaccines. Clin Diagn Virol 1996;5:43-53.
5. Robertson JS, Nicolson C, Riley A-M, Bently M, Dunn G, Corcoran T, et al. Assessing the significance of reverse transcriptase activity in chick cell-derived vaccines. Biologicals 1997;25:403-14.
6. Weissmahr RN, Schupbach J, Boni J. Reverse transcriptase activity in chicken embryo culture supernatants is associated with particles containing endogenous avian retrovirus EAV-0 RNA. J Virol 1997;71:3005-12.
7. Tsang SX, Switzer WM, Shanmugam V, Johnson JA, Goldsmith C, Wright A, et al. Evidence of avian leukosis virus subgroup E and endogenous avian virus in measles and mumps vaccines derived from chicken cells: investigation of transmission to vaccine recipients. J Virol 1999;73:5843-51.
8. WHO Expert Committee on Biological Standardization. Requirements for measles, mumps and rubella vaccines and combined vaccines (live). Requirements for biological substances, no. 47. World Health Organ Tech Rep Ser 1994;840:100-207.
9. Coffin JM, Tsichlis PN, Conklin KF, Senior A, Robinson HL. Genomes of endogenous and exogenous avian retroviruses. Virology 1983;126:51-72.
10. Payne LN. Biology of avian retroviruses. In: Levy JA, editor. The retroviridae. New York: Plenum Press; 1992. p. 299-403.
11. Weiss RA, Frisby DP. Are avian endogenous viruses pathogenic? In: Yohn D, Blakeslee IR, editors. Proceedings of the tenth international symposium for comparative research on leukemia and other diseases. Amsterdam: Elsevier;1982. p. 303-11.
12. Resnick RM, Boyce-Jacino MT, Fu Q, Faras AJ. Phylogenetic distribution of the novel avian endogenous provirus family EAV-0. J Virol 1990;64:4640-53.
13. Dunwiddie C, Faras AJ. Presence of reverse transcriptase-related gene sequences in avian cells lacking endogenous avian leukosis viruses. Proc Natl Acad Sci U S A 1985;82:5097-101.
14. Maudru T, Cooperating units, Peden KWC. Analysis of coded panel of licensed vaccines by polymerase chain reaction-based reverse transcriptase assays: a collaborative study. J Clin Virol 1998;11:19-28.

15. WHO. Reverse transcriptase activity in chicken-cell derived vaccine. Wkly Epidemiol Rec 1998;28:209-12.
16. Thomas PA, Weedon J, Krasinski K Abrams E, Shaffer N, Matheson P, et al. Maternal predictors of perinatal human immunodeficiency virus transmission. Pediatr Infect Dis J 1994;13:489-95.
17. Helfand RF, Gary HE, Atkinson WL, Nordin JD, Keyserling HL, Bellini WJ. Decline of measles-specific immunoglobulin M antibodies after primary measles, mumps and rubella vaccination. Clin Diagn Lab Immunol 1998;5:135-8.
18. Crittenden LB. Retroviral elements in the genome of the chicken: implications for poultry genetics and breeding. Crit Rev Poultry Biol 1991;3:73-109.
19. Mulder J, McKinney N, Christopherson C, Sninsky J, Greenfield L, Kwok S. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. J Clin Microbiol 1994;32:292-300.
20. Lal RB, Heneine W. Testing of human T-lymphotropic virus types I and II: serologic, virologic, and molecular detection. Human T-cell lymphotropic virus type I New York: John Wily and Sons; 1996. p. 167-95.
21. Paul DA, Falk LA, Kessler HA, Chase RM, Blaauw B, Chudwin DS, et al. Correlation of serum HIV antigen and antibody with clinical status in HIV infected patients. J Med Virol 1987;22:357-63.
22. Adkins HB, Brojatsch J, Naughton J, Rolls MM, Pesola JM, Young JA. Identification of a cellular receptor for subgroup E avian leukosis virus. Proc Natl Acad Sci U S A 1997;94:11617-22.
23. Welsh RM, Cooper NR, Jensen FC, Oldstone MBA. Human serum lyses RNA tumor viruses. Nature 1975;257:612-4.
24. Fadly AM. Avian retroviruses. Vet Clin North Am Food Anim Pract 1997;1:71-85.
25. Jacino-Boyce MT, O'Donoghue K, Faras AJ. Multiple complex families of endogenous retroviruses are highly conserved in the genus gallus. J Virol 1992;66:4919-29.
26. Dougherty RM, Harris RJC. Contaminant viruses in two live virus vaccines produced in chicken cells. J Hyg Cambridge 1966;64:1-7.
27. Waters TD, Anderson PS, Beebe GW, Miller RW. Yellow fever vaccination, avian leukosis virus, and cancer risk in man. Science 1972;177:76-77.
28. Levine S, Markham FS. The absence of serologic responses by children and adults to avian leukosis virus in measles vaccine. Archiv fur die gesante Virusforschung 1965;15:305-10.
29. Richman AV, Auliso CG, Jahnes WG, Tauraso NM. Avian leukosis antibody response in individuals given chicken embryo derived vaccines. Proc Soc Exp Biol Med 1972;139:235-7.

A Flea-Associated Rickettsia Pathogenic for Humans

Didier Raoult,* Bernard La Scola,* Maryse Enea,* Pierre-Edouard Fournier,* Véronique Roux,* Florence Fenollar,* Marcio A.M. Galvao,† Xavier de Lamballerie*
* Unité des Rickettsies, CNRS UPRESA 6020, France;
†Ouro Preto Federal University, Brazil

A rickettsia named the ELB agent, or "*Rickettsia felis*," was identified by molecular biology techniques in American fleas in 1990 and later in four patients from Texas and Mexico. We attempted to isolate this rickettsia from infected fleas at various temperatures and conditions. A representative isolate of the ELB agent, the Marseille strain, was characterized and used to develop a microimmunofluorescence test that detected reactive antibodies in human sera. The ELB agent was isolated from 19 of 20 groups of polymerase chain reaction-proven infected fleas. The microimmunofluorescence results provided serologic evidence of infection by the ELB agent in four patients with fever and rash in France (2) and Brazil (2), supporting the pathogenic role of this rickettsia. Our successful isolation of this rickettsia makes it available for use in serologic tests to determine its clinical spectrum, prevalence, and distribution.

Rickettsia are intracellular Proteobacteria associated with arthropods, including body lice, fleas, ticks, and mites (1). *R. typhi*, the agent of murine typhus, is transmitted by rat fleas, *Xenopsylla cheopsis*. In 1990, when cat fleas (*Ctenocephalides felis*) were examined as possible vectors of *R. typhi*, a novel *Rickettsia*-like organism was observed by electron microscopy in midgut epithelial cells of the fleas. The agent, named the ELB agent for the EL Laboratory (Soquel, CA) (2), was detected in 1994 and 2000 by polymerase chain reaction (PCR) in four patients from Texas and Mexico (3,4). The taxonomic position of this organism within the genus *Rickettsia* was assessed by genomic sequence comparison, following the successful amplification of a 17-kDa protein gene fragment from infected flea tissue by PCR with genus-specific primers (5). In addition, the organism was found to be transmitted transovarially in fleas (5) and to be pathogenic in a human patient (6). In 1995, the name "*R. felis*" was proposed for the ELB agent on the basis of its phenotypic characteristics, as well as its clear genotypic differences from other known *Rickettsia* species (6). The organism was provisionally named "*R. felis*" but the name is not formally approved by International Society for Systematic and Evolutionary Biology as no strain was deposited in any official collection. In 1997, the ELB agent was detected in two other flea species in the United States, *C. felis* and *Pulex irritans* (7). Although isolation in tissue culture was reported (3,8,9), contamination with *R. typhi* has hampered subsequent work (6), so no isolate of *R. felis* was available when we began our study.

We describe methods used to cultivate several isolates of the ELB agent and its morphologic, antigenic, and genomic characteristics, as well as the results of a serosurvey with one of our type strains.

Address for correspondence: D. Raoult, Unité des Rickettsies, CNRS UPRESA 6020, Faculté de Médecine, Université de la Méditerranée, 27 Bd Jean Moulin, 13385 Marseille Cedex 05, France; fax: 33-4-91-38-7772; e-mail: Didier.Raoult@medecine.univ-mrs.fr.

Materials and Methods

C. felis fleas (Flea Data Inc., Freeville, NY) were divided into 20 groups of 5. After surface sterilization by a 5-minute immersion in 70% methanol with 0.2% iodine, the fleas were washed in sterile distilled water and frozen in liquid nitrogen. Frozen fleas were macerated with a sterile plastic spatula, suspended in 0.8 mL of culture medium, and injected into shell

vials. Fifty microliters of the suspension was retained for use as template in a *Rickettsia*-specific PCR targeting a fragment of the citrate synthase-encoding gene (*glt*A) (10).

Human embryonic lung fibroblasts (11) or the XTC-2 cell line derived from *Xenopus laevis* (12) were used for isolation by the shell vial centrifugation technique (6). Subconfluent cell monolayers were obtained by incubating the shell vials at 28°C for 48 hours after they were injected with 50,000 cells in 1 mL of Leibowitz-15 medium with L-glutamine and L-amino-acids (GIBCO, Rockville, MD), 5% (v/v) fetal calf serum and 2% (v/v) tryptose phosphate (GIBCO) for XTC-2 cells, and Minimum Essential Medium (GIBCO) supplemented with 2 mM L-glutamine and 10% fetal bovine serum for human embryonic lung fibroblasts. Before injection with the flea extract, the medium was removed by aspiration.

After injection of both cell types with suspensions of five fleas resuspended in 0.8 mL of the corresponding medium, the shell vials were centrifuged at 700 X *g* for 1 hour at 20°C, and the supernatant was discarded. After two washings in sterile phosphate-buffered saline (PBS), 1 mL of fresh medium containing 4 µg/mL cotrimoxazole, an antibiotic used to prevent contamination, was added to the shell vials, which were incubated at 28°C. The cell culture medium was replaced every 7 days for up to 30 days. When rickettsiae could be detected in cells in the discarded medium by Gimenez staining (13), the infected cell monolayer was harvested and spread onto a subconfluent cell monolayer of the same cell line in a 25-cm^2 tissue culture flask, which was also incubated at 28°C.

For further studies, we used our first isolate of the ELB agent, which we named the Marseille strain. We attempted to infect mammalian cell lines with the Marseille isolate on monolayers of Vero, MRC-5, and L-929 cells incubated with 5% CO_2 at 28°C, 32°C, or 37°C, in the same medium as human embryonic lung fibroblasts. The ultrastructure of the Marseille isolate was studied by electron microscopy (14), and the strain was purified as for other rickettsia (15).

To quantify growth of the ELB agent, 1 µL of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilutions of a suspension made of rickettsiae harvested from a 25-cm^2 tissue culture flask and purified from cells were deposited onto 30-well microscope slides (Dynatech Laboratories Ltd., Billingshurt, UK), air dried, fixed with acetone for 10 minutes, and then stained by the Gimenez technique. The number of rickettsiae was estimated visually.

To raise polyclonal antisera against the Marseille isolate, 6- to 8-week-old Balb C mice were injected intraperitoneally with 1 mL of a solution containing v/v 10^6 purified Marseille isolate in PBS and complete Freund's adjuvant. Inoculation was repeated at 10, 20, and 30 days. At 40 days, blood was collected by intracardiac puncture, and sera were stored at –20°C. The same procedure was performed with *R. conorii* (Moroccan strain, ATCC VR 141) and *R. typhi* (Wilmington strain, ATCC VR-144).

To determine the prevalence of antibodies reactive with the organism in the general population in France, serum specimens from 100 French blood donors were tested by microimmunofluorescence against the Marseille strain; 140 serum samples from Brazilian blood donors, which had been sent to our laboratory to estimate the seroprevalence of various rickettsioses, were also tested. Microimmunofluorescence was also used to determine cross-reactivity between our strain of the ELB agent and other rickettsiae. Convalescent-phase serum specimens from 67 patients with epidemiologic, clinical, and serologic evidence of epidemic typhus, 16 patients with murine typhus, and 97 French patients with fever and rash serologically and clinically diagnosed as Mediterranean spotted fever, were tested by microimmunofluorescence for antibodies against *R. rickettsii, R. typhi,* and the Marseille strain. Serum samples from 16 Brazilian patients with unexplained febrile rash were also included in these tests. For microimmunofluorescence, *R. conorii* strain Seven (Malish)*, R. rickettsii* strain R (Bitterroot)*, R. prowazekii* strain Brein L, and *R. typhi* strain Wilmington were grown in Vero cells and purified (11). These antigens and the purified suspension of the Marseille strain described above were applied at separate sites on each well of 30-well microscope slides (Dynatech Laboratories, Ltd.), air dried, and fixed with acetone for 10 min. Microimmunofluorescence tests were performed (16), with immunoglobulin (Ig) G and IgM titers determined separately. To remove antibodies against any host-cell components, antisera were absorbed with XTC-2 or Vero cells before being used in the microimmunofluorescence. Moreover, before detection of IgM, the antisera were absorbed with rheumatoid factor absorbent (Behring-Werke AG, Marburg, Germany). The

antisera were then applied to the fixed antigens at doubling dilutions from 1:4 to 1:2,048 (16).

Analysis of major proteins of the Marseille isolate by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed by using both heated and unheated antigens (17). Major immunogenic proteins were studied by Western blot with purified unheated antigens (14,17).

For PCR amplification and sequencing of gene fragments from macerated flea suspensions and rickettsial isolates, genomic DNA was extracted by using the QIAmp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In addition to the 17-kDa antigen- and citrate synthase-encoding genes determined by Azad et al. (5) and Higgins et al., respectively (3), we amplified and sequenced fragments of the genes encoding the rickettsial outer membrane proteins A and B (*omp*A and *omp*B). Primers were designed within conserved regions of the genes, and amplifications were carried out as described (Table 1,18). Sequencing reactions with these primers were done with the dRhodamine Terminator sequencing kit (PE Applied Biosystems, Warrington, UK). The reaction products were resolved on 5% polyacrylamide gels (Tebu, Le Perray en Yvelines, France) by an ABI PRISM 377 DNA sequencer (PE Applied Biosystems). Amplifications of *R. typhi* by PCR from subcultures of the Marseille strain were performed by using the *glt*A-derived *R. typhi*-specific primers TY1f (5'-TGGGGAACTACCAAGTAGT-3') and TY1r (5'-ACCAGTGCTAATACATGCAA-3') as described to determine the purity of the culture. DNA was extracted as described. DNA from *R. typhi* cultured in Vero cells was used as positive control.

Nucleotide sequences were aligned with sequences from other *Rickettsia* species in GenBank by using the multisequence alignment software CLUSTAL within the BISANCE environment (20). The phylogenetic relationships between our strain and other representative

Table 1. Primers used for PCR amplification and sequencing of the ELB agent strain Marseille-URRWFXCal$_2$

Gene	Primer	Nucleotide sequence (5'-3')	Gene position relative to open reading frame	Reference
17-kDa antigen	17kDF[a]	GCTCTTGCAACTTCTATGTT	31-50	This manuscript
	17KdR[a]	CATTGTTCGTCAGGTTGGCG	464-445	This manuscript
Citrate synthase	CSFEL1[a]	TGATTCAGAATTTGCCGAAT	21-40	17
	CS890r[a]	GCTTTAGCTACATATTTAGG	890-871	17
	CS532r[a]	GCCGCAATGTCTTATAAATATTCT	532-555	This manuscript
	CS1273r[a]	CATAACCAGTGTAAAGCTG	1273-1255	This manuscript
	CS244r[b]	CTTTAATATCATATCCTCGAT	244-224	17
	Rp877P[b]	GGGGACCTGCTCACGGCGG	797-815	18
rOmpB	120-M59[a]	CCGCAGGGTTGGTAACTGC	M59-M41	This manuscript
	120-807[a]	CCTTTTAGATTACCGCCTAA	807-788	This manuscript
	120-607[a]	AATATCGGTGACGGTCAAGG	607-626	This manuscript
	120-1497[a]	CTATATCGCCGGTAATT	1497-1480	This manuscript
	120F-1351[a]	TTTAGGAAACGCTGGTTCTC	1351-1370	This manuscript
	120F-2934[a]	GCGTTAGTTGCGATAATACT	2934-2915	This manuscript
	120-2788[a]	AAACAATAATCAAGGTACTGT	2788-2808	This manuscript
	120-3599[a]	TACTTCCGGTTACAGCAAAGT	3599-3579	This manuscript
	120F-3440[a]	GTTAATGCAACAACTACGGG	3440-3459	This manuscript
	120F-4341[a]	GCATCGAAGAAGTAACGCTG	4341-4322	This manuscript
	120-4232[a]	GGTTTCTCATTCTCTCTATATGG	4232-4254	This manuscript
	120-4879[a]	TTAGAAGTTTACACGGACTTT	4879-4857	This manuscript
	120F-1749[b]	CTATTAGTGGTAATATTGGTAC	1749-1770	This manuscript
	120F-2495[b]	CATGGTCATTACCTGCATTACC	2495-2481	This manuscript
	120-4489[b]	GTCTTCTGACGAAAACTACAA	4489-4509	This manuscript
ROmpA	190-70[a]	ATGGCGAATATTTCTCCAAAA	70-90	18
	190-701[a]	GTTCCGTTAATGGCAGCATCT	701-681	19

[a]Primers used for both PCR amplification and sequencing of the ELB agent strain Marseille-URRWFXCal$_2$.
[b]Primers used only for sequencing.

strains based on the analysis of *glt*A and *omp*B sequences were determined by using the Phylip software (21). The distance matrix generated by DNADIST was determined under the assumption of Jukes and Cantor (22) and used to construct a dendrogram by the neighbor-joining method (23). Two other dendrograms were constructed by using data processing with the maximum-likelihood and parsimony program DNAPARS. Bootstrap replicates were performed by using SEQBOOT and CONSENSE in the PHYLIP software to estimate the node reliability of the trees obtained by the three methods (24).

To improve the sensitivity of PCR and detect rickettsial DNA from serum samples, we designed a nested PCR based on the amplification of the *glt*A gene. DNA was extracted from 200 µL of serum by using the QIAmp blood kit (Qiagen) as recommended by the manufacturer. External primers designed for this purpose were ELB1f (5'-CTGCTTCTTGTCAGTCTAC-3') and ELB1r (5'-GATTTTTTGTTCAGGGTCTTC-3'), and internal primers were ELB2f (5'-GGAATCT TGCGACATCGA-3') and ELB2r (5'-CAGCCTACG GTTCTTGC-3'). The internal primers encompassed a 952-bp *glt*A fragment allowing a reliable identification of most rickettsial species after sequencing (including *R. felis*, *R. conorii*, and *R. rickettsii*). Amplification, sequencing of amplicons, and sequence analysis were done as described. Forty-seven serum samples were tested with this technique, 27 from patients with rickettsial diseases (including those reacting serologically to the ELB agent) and 20 from blood donors used as negative controls.

Results

The ELB agent was detected by PCR amplification of a *glt*A fragment from all the flea suspensions used to inoculate the cell culture monolayers. Initial attempts to isolate the rickettsia in human embryonic lung fibroblasts failed, but growth was observed by Gimenez staining and the rickettsiae were confirmed as the ELB agent by PCR analysis: 100% homology was observed with DNA amplified with the *gltA* sequence of the ELB agent in 19 of 20 supernatants from the XTC-2 cell monolayers grown at 28°C 7 and 14 days after inoculation. *R. typhi* DNA could not be detected by PCR in any of the cell culture supernatants, whereas the primer pair Ty1f and TY1r amplified positive control DNA.

Initial isolation required 14 days, and subsequent passages required 6 days to detect the ELB agent. Cultures of all isolates were easily established in XTC-2 cells from the 19 suspensions. The reference strain, Marseille-URRWFXCal$_2$, has been deposited (accession number I-2363) in the French National Culture Collection (Institut Pasteur, Paris, France) and will be sent to the American Type Culture Collection. Before further studies of this strain, we reconfirmed by PCR that our strain of the ELB agent was not contaminated with *R. typhi*.

The Marseille strain grew most rapidly at 28°C in XTC-2 cells, which died at temperatures ≥32°C (Table 2). Human embryonic lung fibroblasts do not multiply at 28°C, as their optimal growth temperature is 37°C, and therefore the ELB agent could not be cultivated in this cell line. The organism also grew in Vero cells incubated at either 28°C or 32°C but at half the rate of growth observed in XTC-2 cells. The MRC-5 and L-929 cell lines were unable to support permanent growth of the Marseille strain. Electron microscopy showed the rickettsia to be present and free in the cytoplasm but not in the nucleus of the cells (Figure 1).

Table 2. Time required to infect 90% of cells in a 174-cm^2 cell culture flask after inoculation with 5 x10^5 ELB agent strain Marseille-URRWFXCal$_2$

	Incubation temperatures		
Cell type	28°C	32°C	37°C
XTC-2 cells	6 days	ND[a]	ND[a]
Vero cells	14 days	14 days	No
MRC-5 cells	NG[b]	NG[b]	NG[b]
L-929 cells	NG[b]	NG[b]	NG[b]

[a]ND = not done. XTC-2 cells died at temperatures 32°C.
[b]NG = no growth was obtained

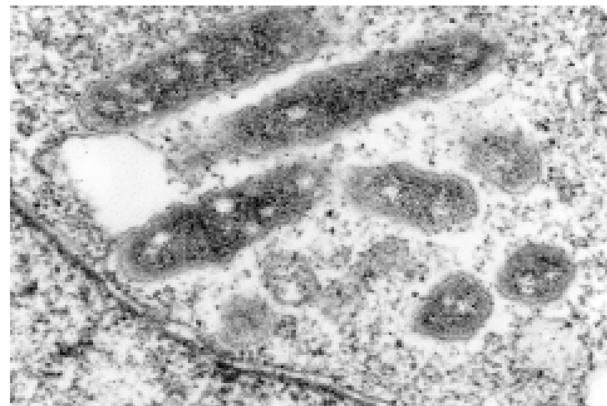


Figure 1. Transmission electron micrograph of the ELB agent in XTC-2 cells. The rickettsia are free in the cytoplasm and surrounded by an electron transparent halo. Original magnification X 30,000.

Four gene fragments were successfully amplified by PCR, and the base sequences of both DNA strands of each segment were determined twice. Nonambiguous sequence data were obtained between bases 51 and 444 of the 17-kDa antigen-encoding gene (394 base pairs), bases 41 and 1236 of *gltA* (1196 bp), bases 91 to 665 of *ompA* (575 bp), and bases 1 to 1236 of *ompB* (1236 bp). Sequences of the 17-kDa protein-encoding gene and *gltA* were 100% homologous with those in GenBank (accession numbers M82878 and U33922, respectively). The GenBank accession numbers for the Marseille strain of the ELB agent nucleotide sequence data reported in this paper are as follows: citrate synthase-encoding gene, AF210692; 17-kDa protein-encoding gene, AF210693; outer membrane protein A-encoding gene, AF210694; and outer membrane protein B-encoding gene, AF2106695. Phylogenetic analysis inferred from the comparison of *gltA* and *ompB* nucleotide sequences with the three analysis methods produced similar organizations. The ELB agent clustered with *R. akari* and *R. australis* (Figure 2).

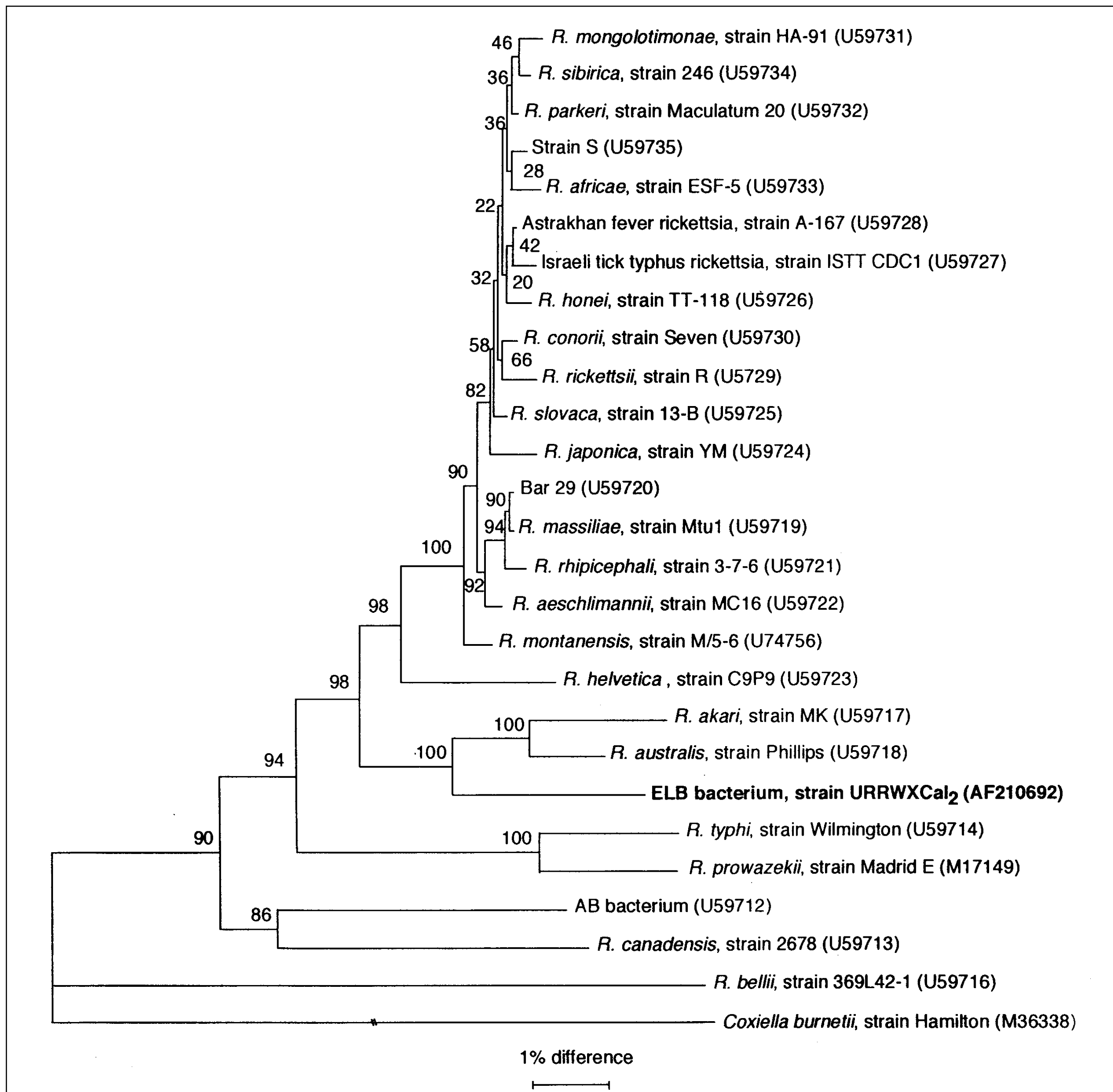


Figure 2. Phylogenetic tree of members of the genus *Rickettsia* inferred from comparison of *gltA* sequences by using the neighbor-joining method. Bootstrap values for the nodes are indicated.

The SDS-PAGE profile of the ELB agent differs from those of *R. conorii* and *R. typhi* (Figure 3). The ELB agent had a high molecular weight protein with a molecular mass of >150 kDa, which was not present in *R. typhi* (Figure 3), as well as a 30-kDa heat-labile protein not present in *R. typhi* or *R. conorii.* Mouse antisera had a 1:1,600 IgG titer against the ELB agent. In Western blots (Figure 3), mouse antisera to the ELB agent reacted strongly with high molecular mass proteins of the agent, another protein of about 30 kDa, and the lipopolysaccharide antigens. Mouse antisera cross-reacted weakly with the high molecular-mass protein antigens of *R. typhi* and *R. conorii*.

None of the serum specimens from the French or Brazilian blood donors had substantial antibody titers against the ELB agent. Of the 67 sera from patients with epidemic typhus, 51 (76%) had antibodies reactive with both *R. prowazekii* and the ELB agent; 66 of these sera had lower titers to the ELB agent than to *R. prowazekii*, and one specimen had the same titer to both organisms. Eleven of the sera from the 16 patients with murine typhus contained antibodies reactive with both *R. typhi* and the ELB agent, but titers were lower against the ELB agent in each specimen, except for one with identical titers to the ELB agent and *R. typhi*. Of the 97 sera from patients with suspected Mediterranean spotted fever, 30 had antibodies to *R. conorii* only, and 67 had antibodies reactive with both *R. conorii* and the ELB agent, with titers identical in one patient and greater against the ELB agent in two patients (patients 1 and 2, Table 3). These two patients, a woman and a man from Marseille, had febrile exanthema in 1995 and 1998, respectively. The other 64 patients had higher antibodies to *R. conorii* than to the ELB agent. Among the Brazilian patients with febrile rash, nine had higher antibody levels to *R. rickettsii*, the agent of Rocky Mountain spotted fever, and the ELB agent; one had higher titers to *R. typhi*; and two had higher titers to the ELB agent (patients 3 and 4, Table 3). These two patients had fever, rash, vomiting, and stupor. Patient 3, in whom the ELB agent was identified by sequencing following nested-PCR amplification

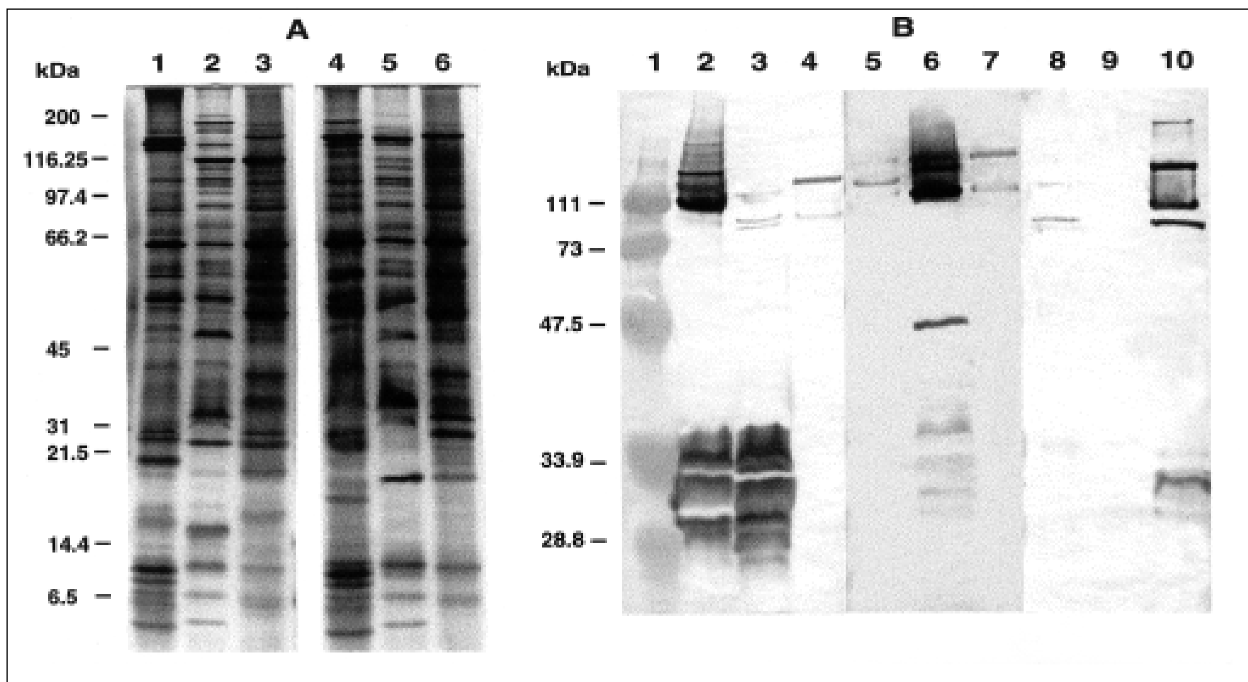


Figure 3. (A) Silver-stained SDS-PAGE of whole-cell protein preparations of *Rickettsia conorii*, the ELB agent and *R. typhi*. Lane 1, *R. conorii*; lane 2. ELB agent; lane 3, *R. typhi*; lane 4, heated *R. conorii*; lane 5, heated ELB agent; lane 6, heated *R. typhi*. Molecular weights are indicated on the left. (B) Western blot of rickettsial proteins probed with various antisera. *R. conorii* antigens (lanes 2, 6, and 10), ELB agent antigens (lanes 3, 7, and 11) and *R. typhi* antigens (lanes 4, 8, and 12) were probed with anti-*R. conorii* (lanes 2 to 4), anti-ELB agent (lanes 5 to 7) and anti-*R. typhi* (lanes 8 to 10) polyvalent mouse antisera. Lane 1: molecular weight marker.

Table 3. Serologic studies of French (patient 1 and 2) and Brazilian (patient 3 and 4) patients with serologic evidence of infection with the ELB agent

Patient	Antibody titers to ELB agent		*Rickettsia prowazekii*		*R. typhi*		*R. conorii*		*R. rickettsii*	
no.	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
1	2,048	64	0	0	0	0	64	64	ND[a]	ND
2	512	32	0	0	0	0	128	32	ND	ND
3	1,024	64	ND	ND	0	64	ND	ND	64	64
4	512	64	ND	ND	0	64	ND	ND	128	64

[a]ND: not done

from a serum sample, had coma, thrombocytopenia 71,000/mL, and aspartate aminotransferase transaminase 75 IU/mL. Three additional Brazilian patients had similar titers against *R. rickettsii* and the ELB agent. Overall, four patients were identified as infected by the ELB agent, and five had equally elevated antibody levels to the ELB agent and another *Rickettsia*.

Of the 47 serum samples tested, only two were positive by the nested PCR. In addition to detecting the ELB agent in patient 3, this technique also identified *R. rickettsii* in a Brazilian patient who had much higher titers to *R. rickettsii* than to the ELB agent. None of the 20 negative controls reacted in the assay.

Conclusions

Several arthropod-borne pathogenic viruses and bacteria grow more rapidly in the laboratory at temperatures lower than human body temperature (25,26). *Rickettsia* are also better adapted for growth at low temperatures. One of the main microbiologic differences between the typhus and spotted fever groups is their optimal growth temperatures. While members of both groups can be maintained in embryonated eggs at 37°C, optimal growth of the typhus group of rickettsiae is 35°C and of the spotted fever group is 32°C (27). The ELB agent has genotypic and phenotypic characteristics typical of the spotted fever group rickettsiae, and our failure to isolate the organism at human body temperature indicates that this relatively high temperature is not optimal for the efficient recovery of the organism, as is observed with other human pathogens (e.g., *Mycobacterium leprae*, *Yersinia* sp.). Our demonstration of the ability of XTC-2 cells to support the growth of the ELB agent indicates that this cell type is an efficient tool to test the growth of other *Rickettsia* species at lower temperatures. This cell line has also proven to be a versatile host for Bunyaviridae, including Bunyaviruses, α-viruses, flaviviruses, and rhabdoviruses (28).

The use of XTC-2 cells proved effective in recovering the ELB agent from fleas, with isolations from 19 of 20 macerated flea samples positive by PCR for the organism. Other spotted fever group rickettsia or arthropod-borne bacteria, such as *Wolbachia* sp. (29) and *Bartonella bacilliformis* (30), may also be cultivated more effectively at lower temperatures by using this cell line.

A pathogenic role of the ELB agent in four patients from Texas and Mexico has been demonstrated by PCR (6,31). However, because serologic tools for the organism were not available, the prevalence of infections by the ELB agent in different areas has yet to be determined. The ELB agent has been found in several species of fleas in the United States, including *C. felis* and *Pulex irritans* (5). These fleas, however, are prevalent worldwide, and we have detected DNA sequences of the ELB agent in Ethiopian fleas independently tested for another purpose. Although we did not obtain Brazilian fleas, we suspect that the ELB agent has a worldwide distribution in fleas.

Human infections with the agent also appear to be widespread, with our results showing that 2 French patients with clinical rickettsial disease and 2 of 16 Brazilian patients with febrile rash had high antibody titers to the ELB agent. Moreover, we confirmed that our PCR serology consistently identified specific sequences of the ELB agent in the serum of one patient; four such cases have been reported (6,30). Our findings indicate that further specific studies are required to determine the distribution of the ELB agent and the prevalence of the agent and associated infection, but this is the first report from cases outside the United States and Mexico.

The characteristics of our reference strain of the ELB agent, Marseille, differ phenotypically

from those reported for the lost *R. felis* strain (6). Our strain does not grow at 35°C or 37°C, and its SDS–PAGE protein profiles and Western blots differed from those reported for *R. felis* and *R. typhi* (3,7,8). Further studies will determine if the reactive 30-kDa heat-labile protein observed on Western blot could be a truncated r*Omp*A protein, as predicted by genomic studies (31). By PCR and sequencing, we identified our isolates as the ELB agent on the basis of 100% *glt*A sequence homology with the sequence available for *R. felis* (5,6). Therefore, discrepancies with previously reported phenotypic findings may result from contamination of *R. felis* cultures with *R. typhi,* which was reported after experiments by the group that described *R. felis* (9). In our experiments based on PCR techniques, however, we found no evidence of contamination of our isolate with *R. typhi*. To avoid confusion between the characteristics of our isolate and those of the previously characterized *R. felis*, we are preparing a formal taxonomic characterization of our isolate of the ELB agent.

In summary, our experiments have demonstrated the usefulness of XTC-2 cells in isolating arthropod-associated microorganisms. This cell culture system allowed us to establish and make available the Marseille strain of the ELB agent. In addition, we have identified likely cases of infection by the ELB agent. The techniques that we describe should facilitate further studies to determine the prevalence and clinical spectrum of infection by this organism in humans.

Acknowledgments

The authors thank J. Georgi of the El Labs in Soquel, California, for providing cat fleas, S.B. Calic and C.B. Chamone for providing Brazilian patients' serum specimens, and P.J. Kelly for review of the translation.

Dr. Raoult is Director of the Unité des Rickettsies, the national reference center for rickettsiosis and WHO collaborative center. The laboratory is mostly involved in the study of emerging and reemerging bacteria and arthropod-borne diseases.

References

1. Raoult D, Roux V. Rickettsioses as paradigms of new or emerging infectious diseases. Clin Microbiol Rev 1997;10:694-719.
2. Adams JR, Schmidtmann ET, Azad AF. Infection of colonized cat fleas, *Ctenocephalides felis* (Bouché), with a rickettsia-like microorganism. Am J Trop Med Hyg 1990;43:400-9.
3. Higgins JA, Radulovic S, Schriefer ME, Azad AF. *Rickettsia felis:* a new species of pathogenic rickettsia isolated from cat fleas. J Clin Microbiol 1996;34:671-4.
4. Zavala-Velasquez JE, Sosa-Ruiz JA, Zavala-Castro J, Jimenez-Delgadillo B, Vado-Solis IE, Sanchez-Elias RA, et al. *Rickettsia felis*: the etiologic agent of three cases of rickettsiosis in Yucatan. Lancet 2000;356:1079-80.
5. Azad AF, Sacci JB, Nelson WM, Dasch GA, Schmidtmann ET, Carl M. Genetic characterization and transovarial transmission of a typhus-like rickettsia found in cat fleas. Proc Natl Acad Sci U S A 1992;89:43-6.
6. Schriefer ME, Sacci JB Jr, Dumler JS, Bullen MG, Azad AF. Identification of a novel rickettsial infection in a patient diagnosed with murine typhus. J Clin Microbiol 1994;32:949-54.
7. Azad AF, Radulovic S, Higgins JA, Noden BH, Troyer JM. Flea-borne rickettsioses: ecologic considerations. Emerg Infect Dis 1997;3:319-27.
8. Radulovic S, Higgins JA, Jaworski DC, Dasch GA, Azad AF. Isolation, cultivation, and partial characterization of the ELB agent associated with cat fleas. Infect Immun 1995;63:4826-9.
9. Radulovic S, Higgins JA, Jaworski DC, Azad AF. In vitro and in vivo antibiotic susceptibilities of ELB rickettsiae. Antimicrob Agents Chemother 1995;39:2564-6.
10. Trilar T, Radulovic S, Walker DH. Identification of a natural cycle involving *Rickettsia typhi* infection of *Monopsyllus sciurorum sciurorum* fleas from the nests of the fat dormouse (*Glis glis*). Eur J Epidemiol 1994;10:757-62.
11. La Scola B, Raoult D. Diagnosis of Mediterranean spotted fever by cultivation of *Rickettsia conorii* from blood and skin samples using the centrifugation-shell vial technique and by detection of *R. conorii* in circulating endothelial cells: a 6-year follow-up. J Clin Microbiol 1996;34:2722-7.
12. Pudney M, Varma MG, Leake CJ. Establishment of a cell line (XTC-2) from the South African clawed toad, *Xenopus laevis*. Experientia 1973;29:466-7.
13. Gimenez DF. Staining rickettsiae in yolk-sac cultures. Stain Technol 1964;39:135-40.
14. Teysseire N, Chiche-Portiche C, Raoult D. Intracellular movements of *Rickettsia conorii* and *R. typhi* based on actin polymerization. Res Microbiol 1992;143:821-9.
15. Eremeeva ME, Balayeva NM, Ignatovich VF, Raoult D. Proteinic and genomic identification of spotted fever group rickettsiae isolated in the former USSR. J Clin Microbiol 1993;31:2625-33.
16. Teysseire N, Raoult D. Comparison of Western immunoblotting and microimmunofluorescence for diagnosis of Mediterranean spotted fever. J Clin Microbiol 1992;30:455-60.
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
18. Roux V, Fournier PE, Raoult D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR amplified DNA of the gene encoding the protein rOmpA. J Clin Microbiol 1996;34:2058-65.

19. Roux V. Phylogenetic analysis and taxonomic relationships among the genus *Rickettsia*. In: Raoult D, Brouqui P, editors. Rickettsiae and rickettsial diseases at the turn of the third millennium. Marseille: Elsevier;1999. p. 52-66.
20. Dessen P, Fondrat C, Valencien C, Munier G. BISANCE: a French service for access to biomolecular databases. Cabios 1990;6:355-6.
21. Felsenstein J. PHYLIP-phylogeny inference package (version 3.2). Cladistics 1989;5:164-6.
22. Jukes TH, Cantor CR. Mammalian protein metabolism. In: Munro HN, editors. Evolution of protein molecules. New York: Academic Press; 1969. p. 121-32.
23. Saitou N, Nei M. The neighbor-joining method: a new method for sequences. J Mol Biol 1987;16:111-20.
24. Brown JKM. Bootstrap hypothesis tests for evolutionary trees and other dendograms. Proc Natl Acad Sci U S A 1994;91:12293-7.
25. Maurin M, Birtles RJ, Raoult D. Current knowledge of *Bartonella* species. Eur J Clin Microbiol Infect Dis 1997;16:487-506.
26. Perry RD, Fetherston JD. *Yersinia pestis:* etiologic agent of plague. Clin Microbiol Rev 1997;10:35-66.
27. Weiss E, Moulder JW. The Rickettsias and Chlamydias. In: Kreig NR, Holt JG, editors. Bergey's manual of systematic bacteriology. Baltimore: Williams & Wilkins; 1984; p. 687-739.
28. Watret GE, Pringle CR, Elliott RM. Synthesis of Bunyavirus-specific proteins in a continuous cell line (XTC-2) derived from *Xenopus laevis*. J Gen Virol 1985;66:473-82.
29. O'Neill SL, Pettigrew MM, Sinkins SP, Braig HR, Andreadis TG, Tesh RB. In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Mol Biol 1997;6:33-9.
30. Bouyer DH, Crocquet-Valdes PA, Walker DH. Expression and size determination of the rOmpa protein of *Rickettsia felis*. Proceedings of the 15th meeting of the American Society for Rickettsiology. Florida: ASR; 2000. p. 61.
31. Roux V, Rydkina E, Eremeeva M, Raoult D. Citrate synthase gene comparison: a new tool for phylogenetic analysis, and its application for the rickettsiae. Int J Syst Bacteriol 1997;47:252-61.

Gastroenteritis in Sentinel General Practices, the Netherlands

Matty A.S. de Wit,* Marion P.G. Koopmans,* Laetitia M. Kortbeek,* Nan J. van Leeuwen,* A.I.M. Bartelds,† and Yvonne T.H.P. van Duynhoven*

*National Institute of Public Health and the Environment, Bilthoven, The Netherlands; †Netherlands Institute of Primary Health Care, Utrecht, the Netherlands

From 1996 to 1999, the incidence of gastroenteritis in general practices and the role of a broad range of pathogens in the Netherlands were studied. All patients with gastroenteritis who had visited a general practitioner were reported. All patients who had visited a general practitioner for gastroenteritis (cases) and an equal number of patients visiting for nongastrointestinal symptoms (controls) were invited to participate in a case-control study. The incidence of gastroenteritis was 79.7 per 10,000 person years. *Campylobacter* was detected most frequently (10% of cases), followed by *Giardia lamblia* (5%), rotavirus (5%), Norwalk-like viruses (5%) and *Salmonella* (4%). Our study found that in the Netherlands (population 15.6 million), an estimated 128,000 persons each year consult their general practitioner for gastroenteritis, slightly less than in a comparable study in 1992 to 1993. A pathogen could be detected in almost 40% of patients (bacteria 16%, viruses 15%, parasites 8%).

In industrialized countries, gastroenteritis incidence remains high, although improved hygiene and treatment have decreased deaths substantially (1,2). Up-to-date estimates of the incidence, disease burden (absence from work or school, bed rest, and use of medication), microbiologic causes, transmission routes, and pathogen sources are necessary to control gastroenteritis effectively and evaluate preventive measures. While numerous studies have addressed the incidence of specific pathogens in selected populations, little is known about the overall incidence and relative contribution of the broad range of pathogens that cause gastroenteritis. In the Netherlands, data on the incidence of gastroenteritis are available from studies in the early 1990s in the general population and in general practices and from continuous laboratory-based surveillance (3,4). The incidence in the general population was estimated to be 450 per 1,000 person years in 1991 (5,6). Patients were tested for *Salmonella* and *Campylobacter* spp., which were detected in 1.5% and 4.6%, respectively. Of these patients, 10% reported visiting their general practitioner (GP), and an additional 10% reported phoning their GP. The incidence of gastroenteritis for which a GP was consulted was estimated to be 15 per 1,000 person years in two regions from 1987 to 1991 (7) and 9 per 1,000 person years in 1992-1993 (8). In these two studies, stool samples were tested for bacterial pathogens; a small subset of these were tested for rotavirus and adenovirus. In most patients (67% in 1987 to 1991, 80% in 1992 to 1993), no pathogen could be detected.

In the years following these studies, new pathogens have been identified, such as *Cyclospora*, and diagnostic methods have been designed for others (Norwalk-like viruses, astrovirus). In addition, to meet the European regulations on protection against zoonotic agents, preventive measures in the veterinary sector have been implemented to decrease *Salmonella* infections in poultry (9-11). Therefore, in 1996, a new study was started among patients consulting a GP. The aims were to estimate the current incidence, study the role of several pathogens, and identify risk factors for

Address for correspondence: Matty de Wit, National Institute of Public Health and the Environment, Department of Infectious Diseases Epidemiology, PO Box 1, 3720 BA Bilthoven, the Netherlands; fax: 31-30-274-4409; e-mail: matty.de.wit@rivm.nl.

microorganism-specific gastroenteritis. Unique to this study, controls were included and stool samples were tested for a broad panel of pathogens. The same network of GPs was used as in the 1992-1993 study, allowing a comparative analysis.

Materials and Methods

The study was performed in cooperation with the network of GPs from the Continuous Morbidity Registration of the Netherlands Institute of Primary Health Care. The network consists of approximately 44 practices that cover 1% of the Dutch population and are representative of it regarding age, gender, regional distribution, and degree of urbanization. All practices reported the number of consultations for gastroenteritis by age group, gender, practice, and week of consultation (reporting study).

Approximately 34 of the practices (33 in 1996; 35 in 1997; 36 in 1998; and 34 in 1999) participated in the case-control study. Patients who consulted a participating GP were asked to complete a questionnaire and submit a stool sample. For each case, a control was recruited on the same day from patients who consulted their GP for nongastrointestinal complaints. Controls were matched by age group ($\leq$11 years, 12 years and older). Thirty-one GPs registered the number of the study package (containing a questionnaire and a stool kit), which was handed out to a participant, on a registration form with age and gender. Participants collected the stool samples and completed the questionnaire at home, and then sent the samples and questionnaires directly to the National Institute of Public Health and the Environment. The questionnaire addressed health status, demographic characteristics, disease burden, clinical manifestations, and risk factors. Stool samples were tested for *Salmonella, Campylobacter, Shigella,* and *Yersinia* by routine culture; for verocytotoxin-producing *Escherichia coli* by polymerase chain reaction for genes for verocytotoxin 1 and verocytotoxin 2 and *eae* gene; *E. coli* O157 by culture on Sorbitol McConkey Agar (12,13); rotavirus and adenovirus by enzyme-linked immunoassay (ELISA) (Rotaclone and Adenoclone from Meridian Diagnostics, Cincinnati, OH); astrovirus by ELISA (IDEIA from DAKO Diagnostics, Cambridgeshire, UK); Norwalk-like viruses by reverse transcriptase-polymerase chain reaction [14]; intestinal parasites by microscopy after fixation in sodium acetate, acetic acid, and formalin; helminthic ova, cysts, and trophozoites by wet film (iodine stained or unstained); for cysts by Ridley concentration (iodine stained); for cysts of *Cryptosporidium*, *Cyclospora*, and *Isospora belli*, by Ridley concentration (Ziehl-Nielsen staining); for trophozoites of protozoa and, to a lesser extent, for cysts, by permanent stained smear (Haemaluin staining) (15-20). Samples from May 1998 until April 1999 were also tested retrospectively for Sapporo-like viruses (21). All stool samples were stored for future investigations. Standardized protocols were used.

The case definition of gastroenteritis used in this study was three or more loose stools in 24 hours; or diarrhea with two additional gastrointestinal symptoms (vomiting, nausea, fever, abdominal cramps, abdominal pain, blood in stool, mucus in stool); or vomiting with two additional gastrointestinal symptoms (diarrhea, nausea, fever, abdominal cramps, abdominal pain, blood in stool, mucus in stool) preceded by a symptom-free period of 2 weeks.

Statistical Analyses

Crude incidence was calculated from the reporting study, corrected for incomplete years. Corrections for incompleteness of participation of GPs and cases and for list inflation (persons no longer belonging to the practice population) were used to calculate an adjusted incidence. List inflation was calculated on the basis of findings in an ongoing, population-based study, performed in the same general practices (22).

The response rate in cases and controls was estimated by using the registration forms. Reported cases and cases in the case-control study were compared by week, practice, age group, and gender to estimate the number of cases in both and each study part. The total cases reported, recruited for the case-control study, or both were used to estimate an incidence corrected for nonresponse. A logistic regression model was used to identify factors independently associated with total response. A variable was excluded if exclusion did not significantly decrease the log likelihood of the model. Because information on the response of recruited cases was too limited to study effects of patients and practice characteristics, only the total of known cases to participating cases was studied in this model.

The effects on incidence of age and gender of the patient, year of study, degree of urbanization,

region of the Netherlands, and participation of the sentinel practice in the case-control study were estimated univariately; independent effects were estimated by using Poisson regression. A variable was excluded from the model if exclusion did not increase the deviance significantly. Cases that did not meet the case definition and controls that did, according to self-reported symptoms in the questionnaire, were excluded from analyses.

Results

The overall incidence of gastroenteritis from May 1996 to April 1999 was 58.0 per 10,000 person years (2,264 cases/390,417 person years) based on the unadjusted number of reported patients of all sentinel practices. A high seasonal peak was observed in the winter of 1996 (maximum in week 9), and lower peaks in 1998 and 1999 (Figure). Summer peaks occurred in all years of the study but varied in period and size.

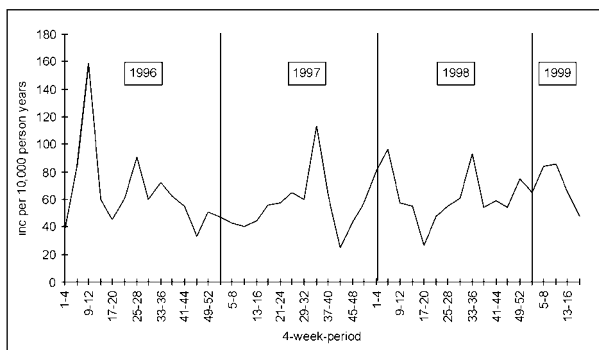


Figure. Incidence of gastroenteritis per 10,000 person years, from reporting of all sentinel practices, the Netherlands, January 1996 to April 1999.

The univariate and multivariate analyses of incidence yielded similar results (Table 1). In the first year of the study (May 1996-April 1997), incidence was lower than in the second (May 1997-April 1998) and third years (May 1998-April 1999). A higher incidence was observed in practices that participated in the case-control study, practices in rural and urban areas (compared to those with intermediate degree of urbanization), and practices in the East, West, and South. The incidence was slightly higher for women than for men and decreased from the youngest age group to the 15- to 24-year age group. The 40- to 64-year-old patients had the lowest incidence. The rate ratios for the different age groups were similar for men and women.

The response of patients was 78% (695 questionnaires completed out of 888 cases registered on registration forms). A total of 2,553 cases were reported, recruited for the case-control study, or both. Of these, 1,138 (45%) patients were recruited, 888 (35%) participated in the case-control study, and 2,165 (85%) were reported. The incidence corrected for incomplete participation of patients and GPs was 77.7 per 10,000 person years (2,553 cases/328,438 person years), according to data from GPs participating in the case-control study. The estimated list inflation was 2.5%. Adjusting the denominator for list inflation yielded a final incidence of 79.7 per 10,000 person years (2,553 cases/320,227 person years).

The percentage of patients recruited by GPs decreased during the study and was higher in the North and South (Table 2). The highest participation rate was for patients 25-64 years of age, mainly because a higher proportion were recruited. Participation was relatively low for 15- to 24-year-old patients.

The response of controls who were recruited was 73% (554 questionnaires completed out of 765 study packages registered). The lowest response rates for controls (42%) were found in the 10- to 19-year-old age group. The response for female controls was slightly higher than for male controls (78% versus 68%). Participation was highest for controls recruited in 1996 (82%) and lowest in 1999 (63%).

Case-Control Study

Population of Participating Practices

The distribution of age, gender, and degree of urbanization in the study population was similar to that of the Dutch population. The North was overrepresented (20% in participating practices, 10% in Dutch population); the West was underrepresented (32% in practices vs. 44% in the population).

Cases and Controls

From May 1996 until April 1999, 985 cases and 717 controls returned a questionnaire to the National Institute of Public Health and the Environment. Of these, 878 cases and 581 controls could be included in the analyses. Stool samples were examined from 857 (98%) patients and 574 (99%) controls. The median age of patients (29 years) was significantly lower than

Table 1. Univariate and multivariate Poisson regression analyses of the incidence per 10,000 person years

Incidence per 10,000 person years	Crude incidence	Univariate Poisson regression		Multivariate Poisson regression	
		RR[a]	95% CI[a]	RR	95% CI
Study year					
May 96-Apr 97	52.4	1.00	-	1.00	-
May 97-Apr 98	62.4	1.19	1.08-1.32	1.19	1.08-1.32
May 98-Apr 99	65.6	1.25	1.13-1.38	1.23	1.11-1.36
Participation in case-control study					
Yes	65.8	2.51	2.13-2.97	2.71	2.28-3.22
No	26.2	1.00	-	1.00	-
Urbanization					
Low	57.0	1.04	0.92-1.17	1.18	1.04-1.34
Intermediate	55.5	1.00	-	1.00	-
High	88.9	1.62	1.47-1.79	1.25	1.11-1.41
Region					
North	28.7	0.39	0.34-0.45	0.35	0.29-0.40
East	71.3	0.97	0.88-1.07	0.93	0.83-1.05
West	76.0	1.00	-	1.00	-
South	55.5	0.75	0.68-0.84	0.78	0.69-0.88
Gender					
Male	56.8	1.00	-	1.00	-
Female	63.4	1.12	1.03-1.21	1.12	1.04-1.22
Age (in years)					
<1	360.4	5.53	4.49-6.82	5.54	4.50-6.83
1-4	221.5	3.40	2.94-3.94	3.36	2.90-3.89
5-14	65.1	1.00	-	1.00	-
15-24	51.7	0.79	0.67-0.94	0.79	0.67-0.93
25-39	55.9	0.86	0.75-0.98	0.84	0.73-0.97
40-64	36.5	0.56	0.48-0.65	0.56	0.48-0.65
≥65	47.7	0.73	0.62-0.87	0.72	0.61-0.85

[a]RR = relative risk; CI = confidence interval.

that of controls (37 years). Gastrointestinal complaints of longer than 1 month were more frequent in patients (31.5%) than in controls (7.3%). The percentage of patients (18%) under treatment of a specialist was similar to that of controls, as was the number of GP consultations in the last 3 months (1 consultation). Patients were slightly more often born outside the Netherlands than controls, and the educational level of patients was higher than that of controls (both not statistically significantly).

Clinical Symptoms of Patients

Almost all patients reported loose stools (Table 3). Other commonly reported symptoms were frequent stools, abdominal pain, abdominal cramps, and nausea. Only 12 (1.4%) patients reported vomiting and no diarrhea. In cases with a higher frequency of stools than normal, the median of the maximum frequency was 6 times in 24 hours (first quartile-third quartile: 4-8 times in 24 hours). The median duration of symptoms before a GP was consulted was 6 days (first quartile to third quartile: 3-20 days); 20% of patients had been symptomatic for more than 4 weeks before consulting a GP.

Bed rest was required for 47% of patients for a median of 2 days (mean 3.1 days); 41% of the children who regularly visited a day-care center had to stay home for a median of 3 days (mean 3.2 days), 58% of schoolchildren were absent from school for a median duration of 3 days (mean 3.8 days), and 60% of working patients were absent from work for a median duration of 2 days (mean 3.1 days). In 8% of cases, someone had to miss school or work for a median of 1.5 days (average 2.0 days) to care for the patient; 56% of patients used medication for gastroenteritis: 4% used antibiotics, 18% analgesics, 27% antidiarrheic medication, 10% oral rehydration solution, and 21% additional medications.

Symptoms and Diagnoses of Controls

A small proportion of controls (6.7%) reported consulting a GP for gastrointestinal symptoms when they were recruited for the study but did

Table 2. Multivariate logistic regression analyses of the selection of participating cases from all cases that were reported or participated in the case-control study, the Netherlands

	Recruited or reported	% Recruited by GP[a]	% Part. cases[b]	% Total response	OR[c] for response	95% CI[d]
Gender					ni[e]	
Male	1,178	45	79	35		
Female	1,370	44	78	35		
Age (yrs)						
0	123	34	76	26	0.84	0.51-1.37
1-4	452	40	76	31	0.89	0.63-1.25
5-14	342	36	82	30	0.85	0.59-1.21
15-24	265	51	65	33	1.00	-
25-39	621	51	76	39	1.30	0.95-1.78
40-64	487	50	85	42	1.50	1.08-2.07
65+	254	35	92	32	0.89	0.61-1.30
Urbaniztion					ni	
Low	305	34	94	32		
Intermediate	1,654	46	79	37		
High	582	45	72	32		
Region						
North	281	60	83	49	2.16	1.63-2.87
East	586	33	84	28	0.86	0.68-1.08
West	1,034	41	73	30	1.00	-
South	642	53	81	43	1.69	1.36-2.08
Year of study						
May 96-Apr 97	804	59	80	47	1.00	
May 97-Apr 98	841	40	81	32	0.50	0.43-0.65
May 98-Apr 99	889	34	77	26	0.40	0.33-0.50
Total	2,553	45	78	35	-	-

[a]GP - general practitioner
[b]part = participating.
[c]OR = odds ratio for participation (defined as a case questionnaire received at the National Institute of Public Health and the Environment).
[d]CI = confidence interval.
[e]ni = not included in the logistic regression model.

Table 3. Self-reported symptoms of patients, Netherlands case-control study

	No. of cases (N=878)	% of cases
Loose stools	861	98.1
Frequent stools[a]	387	78.2
Abdominal cramps	679	77.3
Abdominal pain	673	76.7
Nausea	536	61.0
Vomiting	359	40.9
Fever	335	38.2
Mucus in stool	304	34.6
Blood in stool	97	11.0

[a]More frequent than normal as perceived by the respondent.

not meet the case definition; 14% consulted for other reasons (e.g., to pick up a prescription, routine physical examination, accompanying a relative); most controls gave no information (43%) or had consultations for other symptoms (36%).

Pathogens

The most frequently detected pathogens in cases were *Campylobacter*, *Giardia lamblia*, rotavirus, Norwalk-like viruses, and *Salmonella* (Table 4). Bacterial pathogens were found almost solely in cases, except for *Yersinia* and verocytotoxin-producing *E. coli*. All isolated *Yersinia* spp. were nonpathogenic serotypes. The verocytotoxin-producing *E. coli* serotypes found in controls were different from those found in patients but included pathogenic types, such as O26. *E. coli* O157 K-H- was isolated from one case. The percentages of *Campylobacter, Salmonella,* and *Salmonella* Enteritidis did not differ significantly over the study years. Viral pathogens were found in 1% to 5% of patients and in a small percentage of controls. The possibly nonpathogenic parasite *Dientamoeba fragilis* and the nonpathogenic parasite *Blastocystis hominis* were common and were found more frequently in controls than in patients.

Table 4. Microorganisms detected in patients and controls

	Patients (N=857)		Controls (N=574)	
	N	%	N	%
Salmonella spp.	33	3.9	1	0.2
S. Enteritidis	12	1.4	0	0.0
S. Typhimurium	11	1.3	1	0.2
other *Salmonellae*[a]	9	1.1	0	0.0
Campylobacter spp.	89	10.5	3	0.5
C. jejuni	77	9	3	0.5
C. coli	7	0.8	0	0.0
other species	5	0.6	0	0.0
Yersinia spp.	6	0.7	6	1.1
Y. enterocolitica[b]	5	0.6	5	0.9
Y. frederiksenii[c]	1	0.1	1	0.2
Shigella spp.	1	0.1	0	0.0
S. flexneri	1	0.1	0	0.0
VTEC[d]	4	0.5	3	0.6
Rotavirus	45	5.3	8	1.4
Adenovirus	19	2.2	2	0.4
Astrovirus	13	1.5	2	0.4
Norwalk-like viruses	43	5.0	6	1.1
Sapporo-like viruses[e]	5	2.1	1	0.6
Giardia lamblia	46	5.4	19	3.3
Entamoeba histolytica / dispar	9	1.1	4	0.7
Cryptosporidium	18	2.1	1	0.2
Cyclospora	1	0.1	1	0.2
(Possibly) nonpathogenic microorganisms				
Dientamoeba fragilis	88	10.3	84	14.6
Blastocystis hominis	185	21.7	172	30.0
Endolimax nana	14	1.6	14	2.4
Enteromonas hominis	2	0.2	1	0.2
Chilomastix mesnili	1	0.1	0	0.0
Entamoeba hartmanni	0	0.0	2	0.4
Enterobius vermicularis	0	0.0	1	0.2
Iodamoeba butschlii	0	0.0	1	0.2

[a]*S.* Heidelberg, *S.* Thompson, *S.* Kottbus, *S.* Montevideo, *S.* Manhattan, *S.* Anatum, *S.* Arizona, *Salmonella* group B (not fully typeable).

[b]Serotypes of *Y. enterocolitica* in patients: O6,31 (biotype 1a), O6,30; O6,30 (biotype 1A), one not typeable, one not typed. In controls: two O4 (biotype 1A); O65 (biotype 1A), O7,8 (biotype 1A); one not typeable.

[c]Serotypes of *Y. frederiksenii*: O16a,58 (case); O16AB,29 (control).

[d]VTEC=verocytotoxin-producing *Escherichia coli*: positive for *eae* gene and genes for either SLT1 or SLT2. VTEC serotypes in cases: O157 K-H-, O98 K-, O145 K-, one not typeable. In controls: O26, O145 K-, one not typeable

[e]Sapporo-like viruses have only been tested for the last study year (May 98–Apr 99; 240 cases and 160 controls). Genotypes in cases: 4 Sapporo, 1 London, genotype in control: Sapporo.

In 37.5% of patients and 9.8% of controls, a pathogen could be detected (excluding possibly nonpathogenic microorganisms, Table 4). From May 1998 until May 1999, these percentages were increased by Sapporo-like viruses with 1.7% in patients and 0.6% in controls. When *D. fragilis* was considered a pathogen, these percentages were 44.1% and 22.9%, respectively. The percentage in which a pathogen was found was similar for the 50 controls who reported gastrointestinal symptoms (but did not meet the case definition) and controls without gastrointestinal symptoms.

Most stool samples of patients were received at the National Institute within 1 day of collection (57%), 21% after 2 days, 9% after 3 days, 7% after 4 days, 3% after 5 days, and 2% after ≥6 days. No significant differences were noted in the percentage of samples in which a pathogen was detected for different postal delays. For *Campylobacter*, however, a decreasing trend in the proportion of positive cases was observed with an increasing postal delay: 1 day: 12% positive, 2 days: 10%, 3 days: 9%, 4 days: 6%, 5 days 5%, 6 days or more: no *Campylobacter*.

Discussion

Incidence and Participation Rate

This is the first GP-based national study in the Netherlands covering the role of a wide range of microorganisms in a representative population of gastroenteritis patients and controls. Only England has conducted a similar study.

The incidence of gastroenteritis in general practices was estimated at 79.7 per 10,000 person years, suggesting that in the Netherlands, 1 in every 125 persons, or 128,000 persons, will seek physician care for gastroenteritis every year. This estimate was adjusted for list inflation and partially for nonparticipation (15%) of GPs but not for the number of underascertained cases absent from both study components. In a similar study in England, underascertainment was estimated to be 36% (23). The correction for list inflation (2.5%) in our study should be considered a minimum estimate because only patients who were actively reported as no longer belonging to the general practice population were counted; by contrast, the more active approach in England (searching medical records of nonrespondents)

yielded an estimate of 10% (23). Consequently, the incidence from our study must be considered a conservative, but the best available, estimate.

A previous estimate of the incidence of gastroenteritis in a GP-based study in 1992 to 1993 in the Netherlands was somewhat higher at 90 cases per 10,000 person years (not corrected for list inflation) (8). Since the percentages of *Salmonella* and *Campylobacter* have also decreased since 1992-1993 (from 5% to 4% and from 14% to 10%, respectively), the decrease in incidence could partially be due to preventive measures in the poultry industry that focused on *Salmonella* Enteritidis, but as expected, also caused a slight decrease in *Campylobacter* infections in poultry. Nevertheless, these measures were not fully implemented until April 1997, and therefore a decreasing trend in the incidence within the study period would be expected, which was not observed for gastroenteritis as a whole, nor for the percentages positive for *Salmonella* and *Campylobacter*. Several other factors might have contributed to this decrease, such as a more widespread use of Hazard Analysis and Critical Control Points in the food production industry, greater awareness and knowledge in the population about foodborne infections, and a change in consultation behavior because of GPs' increasing deferral policy for gastroenteritis.

The low incidence in the first study year was presumably due to the absence of a winter peak in 1996 to 1997. Such peaks coincide with an increase in infections with enteric viruses, such as astrovirus, rotavirus, and calicivirus (24-26). Therefore, the variation in incidence in different years likely is due to the annual fluctuation of the peaks in viral pathogens.

Our data indicate an incidence almost fourfold lower than the incidence in England. The GP-based incidence of gastroenteritis estimated for England in a study from 1993 to 1996 was 330 per 10,000 person years after correction for list inflation and underreporting (27). In Wales in 1992, an incidence of 244 per 10,000 person years was found (28). Most likely, gastroenteritis patients' higher consultation rate in England accounts for the difference: approximately 1 in 6 gastroenteritis case-patients in England consult a GP, whereas an estimated 1 patient in 10 to 1 patient in 50 does in the Netherlands (5,6,8). A lower consultation rate could be explained by Dutch GPs' policy of deferring gastroenteritis cases. In the Dutch Guidelines for Acute Diarrhea, a consultation by telephone is considered adequate for uncomplicated acute diarrhea and can be dealt with by the GP's assistant (29). Of the 19 participating GPs who completed a questionnaire at the end of our study, 12 (63%) reported fully or partially discouraging patients with gastroenteritis from visiting their practice. In England, a deferral policy for consultations for gastroenteritis is not common.

The incidence of gastroenteritis was independently associated with degree of urbanization and geographic region. The incidence was the lowest in the North, as was found in the study in 1992 to 1993 (8). This cannot be explained by a lower response rate (Table 2). A higher incidence was found in areas with a high or low degree of urbanization than in areas with an intermediate degree of urbanization. Other studies also report a higher incidence in urban areas (30). Possibly, a high population density causes an increased risk for person-to-person transmission. In rural areas, contact with animals, manure, and raw products could be more frequent than in more urbanized regions, possibly leading to higher levels of exposure to zoonotic pathogens. An analysis of risk factors for each pathogen will be published after an ongoing population-based study is completed. The differences in incidence could also reflect differences in consultation behavior.

Patient-related factors that were independently associated with incidence were patient's gender and age. The incidence was higher for women than for men and was clearly higher in the youngest age groups, consistent with other studies (5,8,28,31-33). Although several studies have reported a higher disease burden for the elderly, no increase in the incidence of gastroenteritis was observed in our study (1). Possibly, the higher risk for gastroenteritis for the elderly is limited to the relatively weaker persons living in nursing homes (25). Because these homes usually have their own GP, the proportion of persons living in nursing homes could be underrepresented in this study.

Pathogens

Bacteria were detected in 16.3% of patients, viruses in 15.4%, and pathogenic parasites in

8.3%. The higher participation of the northern region might have increased the percentage of *Salmonella* Typhimurium because *S*. Typhimurium is predominant over *S*. Enteritidis in this region, in contrast to the rest of the Netherlands (34). The order of relative importance of pathogens in general practices was similar for the Netherlands and England, although the percentages positive for viruses and bacteria in this study were slightly lower than in England and the percentages positive for parasites were slightly higher. Because bacteria and viruses are more often detected in patients with acute diarrhea, whereas parasites tend to cause less fulminant but intermittent and long-lasting symptoms that might lead to delayed consultation, these differences could be due to the exclusion of persons with symptoms lasting longer than 2 weeks in the English study (35,36). In our study, 32% of patients had symptoms >2 weeks' duration (36). In addition, we used formalin-fixed material to detect parasites, and four different preparations were examined, which increased the sensitivity of microscopy examination, compared to the use of nonfixed material and the examination of three different preparations in the English study (37). The lower participation of patients in the last 2 years of the study might have caused an underestimate of viral pathogens because the seasonal viral peak was relatively low in the first year (24,38). In spite of a less sensitive method of testing for Norwalk-like viruses (electron microscopy in England versus reverse transcriptase-polymerase chain reaction in the Netherlands), the percentage of stool samples positive for Norwalk-like viruses is higher in England (36). The low response for the younger age groups might also have reduced the percentage of rotavirus, Sapporo-like viruses, and to a lesser degree Norwalk-like viruses, which are most common in young children (24). Differences in the proportion of specific pathogens in the English study and in our study may also be explained by differences in consultation rates. A lower threshold for consulting a GP might increase the proportion of pathogens that cause relatively mild gastroenteritis, such as Norwalk-like viruses.

Parasites that were (possibly) nonpathogenic were more frequently found in controls than in patients. A more detailed study of differences between patients and controls with these parasites might identify factors related to the development of disease after infection with these parasites.

Diagnostic Deficit

In spite of a diagnostic panel that included most of the known pathogens that can cause gastroenteritis, the percentage of patients in which no pathogen could be detected was 61% (including Sapporo-like viruses in 1998 and excluding *D. fragilis* and nonpathogenic parasites). Some cases could be noninfectious because an exclusive distinction between infectious and noninfectious intestinal disease cannot be made clinically. The high percentage of patients with chronic gastrointestinal symptoms suggests that a substantial proportion might not be infectious but an expression of other illnesses, such as inflammatory bowel disease. The symptoms might also be caused by intestinal microorganisms not included in this study or not yet known. Several pathogens, such as *Campylobacter*, verocytotoxin-producing *E. coli*, and torovirus have only recently been recognized as a cause of gastroenteritis, and it is likely that new pathogens will be added to this list (39). In addition, we did not screen for some pathogens, such as some pathogenic *E. coli* (e.g., enterotoxigenic *E. coli*, enteroinvasive *E. coli*) and bacterial toxins (*Bacillus* spp, *Clostridium difficile* cytotoxin, *C. perfringens* enterotoxin), which were associated with 15% and 6% of the cases, respectively, in the English study (36). However, all stool samples from our study were stored and are currently being tested for Sapporo-like viruses and can be tested for other pathogens. Also, intestinal symptoms can be caused by nonintestinal infections, such as influenza, not included in our study. Approximately one quarter of gastroenteritis cases in an American population-based study coincided with respiratory disease (31). Detection of pathogens in the study is also influenced by logistics factors and the sensitivity of the testing method. The timing of sampling was not ideal in many cases; because of the relatively long patient delay, some pathogens might no longer have been excreted in the stool at the time of sampling. Some pathogens, such as *Campylobacter,* do not grow in culture after a long delay in shipping. At present, we are conducting a study on gastroenteritis in the community to elucidate the role of sampling time and referral behavior on pathogen-specific incidence.

Acknowledgments

We thank the participating general practitioners and the Netherlands Institute for Primary Health Care for their indispensable cooperation in the data collection; Jan Vinjé for his work on the diagnostic testing methods and support; Joke Admiraal, Denise Hoek, Nahid Nozari, Hanneke Deijl, Petra de Bree, Jeroen Meijer, and Sandy Altena for their excellent assistance in performing the diagnostic tests; Isabel Araya Segovia for administrative support; Martien Borgdorff for his work in the design and start of the study; and Nico Nagelkerke for his advice on data analyses.

Ms. de Wit works as an epidemiologist at the Department of Infectious Disease Epidemiology of the National Institute of Public Health and the Environment in the Netherlands. Her work focuses on the epidemiology of gastroenteritis in the Netherlands at the hospital, general practice, and community levels.

References

1. Guerrant RL, Hughes JM, Lima NL, Crane J. Diarrhea in developed and developing countries: magnitude, special settings and etiologies. Rev Infect Dis 1990;12:S41-50.
2. Bern C, Martines J, de Zoysa I, Glass RI. The magnitude of the global problem of diarrhoeal disease: a ten-year update. Bull World Health Organ 1992;70:705-14.
3. Esveld MI, van Pelt W, van Leeuwen WJ, Banffer JRJ. Laboratorium Surveillance Infectieziekten. RIVM report no. 968902002. Bilthoven, the Netherlands: RIVM; June 1996.
4. Sprenger MJW, Schrijnemakers PM, Wijgergangs LM, Talsma E. Infectious Diseases Surveillance and Information System (ISIS). International Conference on Emerging Infectious Diseases; 1998 Mar 8-11; Atlanta. p. 123.
5. de Wit MAS, Hoogenboom-Verdegaal AMM, Goosen ESM, Sprenger MJW, Borgdorff MW. A population-based longitudinal study on the incidence and disease burden of gastroenteritis and *Campylobacter* and *Salmonella* infection in four regions of the Netherlands. Eur J Epidemiol 2000;16:713-8.
6. Hoogenboom-Verdegaal AMM, Jong JC, During M, Hoogenveen R, Hoekstra JA. Community-based study of the incidence of gastrointestinal disease in the Netherlands. Epidemiol Infect 1994;112:481-7.
7. Hoogenboom-Verdegaal AMM, Goosen ESM, During M, Engels GB, Klokman-Houweling JM, Laar MJW van de. Epidemiologisch en microbiologisch onderzoek met betrekking tot acute gastro-enteritis in de huisartsenpeilstation in Amsterdam en Helmond, 1987-1991. RIVM report no. 149101011. Bilthoven, the Netherlands: RIVM; 1994.
8. Goosen ESM, Hoogenboom-Verdegaal AMM, Bartelds AIM, Sprenger MJW, Borgdorff MW. Incidentie van gastro-enteritis in huisartsenpeilstations in Nederland, 1992-1993. RIVM report no. 149101012. Bilthoven, the Netherlands: RIVM; 1995.
9. Council directive 92/117/EEC, concerning measures for protection against specified zoonoses and specified zoonotic agents in animals and products of animal origin in order to prevent outbreaks of food-borne infections and intoxications. 1992 Dec. Productschap Vlee, Vleesen Eieren. Rijswijk: PVE; 1997a.
10. Productschap Vee, Vlees en Eieren (PVE). Plan van aanpak preventie en bestrijding Salmonella in de eiersector. Rijswijk, the Netherlands: PVE; 1997a.
11. Productschap Vee, Vlees en Eieren (PVE). Plan van aanpak Salmonella en Campylobacter in de pluimveevleessector. Rijswijk, the Netherlands: PVE; 1997b.
12. Smith HR, Scotland SM. Isolation and identification methods for *Escherichia coli* O157 and other verotoxin producing strains. J Clin Pathol 1993;46;10-17.
13. Edwards and Ewing. Identification of Enterobacteriaceae. 4th ed. New York: Elsevier Science Publishing Co., Inc.; 1986.
14. Vinjé J, Koopmans MPG. Molecular detection and epidemiology of small round-structured viruses in outbreaks of gastroenteritis in the Netherlands. J Infect Dis 1996;174:610-15.
15. Polderman AM, Rijpstra AC, editors. Medische parasitologie, handleiding bij laboratorium diagnostiek. Bohn Stafleu Van Loghum, the Netherlands; Houten/Zaventem; 1993; 218:146-7.
16. Belding DL, editor. Textbook of parasitology. 3rd ed, New York: Appleton-Century-Crofts; 1965. p. 1374.
17. Ash LR, Orihel TC, editors. Atlas of human parasitology. 2nd ed. Chicago: American Society of Clinical Pathologists Press; 1984. p. 212.
18. Yang J, Scholten T. A fixative for intestinal parasites permitting the use of concentration and permanent staining procedures. Am J Clin Pathol 1977;67:300-4.
19. Garcia LS, section editor. Parasitology. Section 7.1-7.4. In: Isenberg HD, editor. Clinical microbiology procedures handbook. Washington: American Society for Microbiology.
20. van Gool T, Mank TG. Fixatives and permanent stains in the laboratory diagnosis of intestinal protozoal infections. Haarlem, the Netherlands: Van Gool and Mank; 1999.
21. Vinjé J, Deijl H, van der Heide R, Lewes D, Hedlund K-O, Svensson L, et al. Molecular detection and epidemiology of Sapporo-like viruses. J Clin Microbiol 2000;38:530-6.
22. Abbink F, Duynhoven YTHP, de Wit M, Koopmans MPG, van Leeuwen WJ, Kortbeek LM. A population cohort study with a nested case-control study: a study design to estimate the incidence and aetiology of gastroenteritis (GE) in the Netherlands. Proceeding 4th World Congress of Foodborne Infections and Intoxications. 1998; Berlin, Germany. p. 766-9.
23. Sethi D, Wheeler J, Rodrigues LC, Fox S, Roderick P. Investigation of under-ascertainment in epidemiological studies based in general practice. Int J Epidemiol 1999;28:106-11.
24. Koopmans MPG, van Asperen IA. Epidemiology of rotavirus infections in The Netherlands. Acta Paediatr Suppl 1999;426:31-7.
25. Vinjé J, Altena SA, Koopmans MPG. The incidence and genetic variability of small round-structured viruses (SRSV) in outbreaks of gastroenteritis. J Infect Dis 1997;176:1374-8.
26. Borgdorff MW, Koopmans MPG, Goosen ESM, Sprenger MJW. Surveillance of gastroenteritis [letter]. Lancet 1995;346:842-3.

27. Wheeler JG, Sethi D, Cowden JM, Wall PG, Rodrigues LC, Tompkins DS, et al. Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. Br Med J 1999;318:1046-50.
28. Palmer S, Houston H, Lervy B, Ribeiro D, Thomas P. Problems in the diagnosis of foodborne infections in general practice. Epidemiol Infect 1996;117:479-84.
29. Nederlands Huisartsen Genootschap [Netherlands General Practitioners Society]. Standaard acute diarree. Huisarts en Wetenschap 1993;36(9).
30. Roderick P, Wheeler J, Cowden J, Sockett P, Skinner R, Mortimer P, et al. A pilot study of infectious intestinal disease in England. Epidemiol Infect 1995;114:277-88.
31. Monto AS, Koopman JS. The Tecumseh study: Occurrence of acute enteric illness in the community. J Epidemiol 1980;112:323-33.
32. Skirrow MB. A demographic survey of *Campylobacter, Salmonella* and *Shigella* infections in England. Epidemiol Infect 1987;99:647-57.
33. Simpson GE, Nagy GS, Fincks ES. A survey of acute gastroenteritis in general practice. Med J Aust 1969;2:633-5.
34. Giessen AW, van Leeuwen WJ, van Pelt W. Chapter 8: *Salmonella enterica* Serovar Enteritidis in the Netherlands: epidemiology, prevention and control. In: Saeed AM, editor. *Salmonella enterica* Serovar Enteritidis in humans and animals. Epidemiology, pathogenesis, and control. Ames (IA): Iowa State University Press;1999. p. 71-80.
35. Mank TG, Polderman AM, Zaat JOM, Roggeveen C, van Eijk JThM, Deelder AM. Persistent diarrhea in a general practice population in the Netherlands: prevalence of protozoal and other intestinal infections. In: Thesis intestinal protozoa and diarrhea in general practice. Haarlem, the Netherlands: Jos Mathôt BV; 1997. p. 47-64.
36. Tompkins DS, Hudson MJ, Smith HR, Eglin RP, Wheeler JG, Brett MM, et al. A study of infectious intestinal disease in England: microbiological findings in cases and controls. Commun Dis Public Health 1999;2:108-13.
37. Mank TG, Zaat JOM, Blotkamp J, Polderman AM. Comparison of fresh versus Sodium Acetate Acetic Acid Formalin preserved stool specimens for diagnosis of intestinal protozoal infections. Eur J Clin Microbiol Infect Dis 1995;14:1076-81.
38. de Wit MAS, Koopmans MPG, van der Blij JF, Duynhoven TYHP. Hospital admissions for rotavirus infection in the Netherlands. Clin Infect Dis 2000;31:698-704.
39. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerg Infect Dis 1999;5:607-25.

Active Bacterial Core Surveillance of the Emerging Infections Program Network

Anne Schuchat,* Tami Hilger,* Elizabeth Zell,* Monica M. Farley,† Arthur Reingold,‡ Lee Harrison,§ Lewis Lefkowitz,¶ Richard Danila, Karen Stefonek,†† Nancy Barrett,‡‡ Dale Morse,§§ and Robert Pinner,* for the Active Bacterial Core Surveillance Team of the Emerging Infections Program Network**

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †Georgia Emerging Infection Program (Georgia Department of Human Resources, Division of Public Health, Emory University School of Medicine, and the Atlanta Veterans Administration Medical Center) Atlanta, Georgia, USA; ‡California Department of Health Services and University of California Berkeley School of Public Health, Berkeley, California, USA; §Maryland Department of Health and Mental Hygiene and Johns Hopkins University School of Public Health, Baltimore, Maryland, USA; ¶Tennessee Department of Health and Vanderbilt University Medical Center, Nashville, Tennessee, USA; **Minnesota Department of Health, St. Paul, Minnesota, USA; ††Oregon Department of Human Resources, Portland, Oregon, USA; ‡‡ Connecticut Department of Public Health, Hartford, Connecticut, USA; and §§New York State Department of Health, Albany, New York, USA

Active Bacterial Core surveillance (ABCs) is a collaboration between the Centers for Disease Control and Prevention and several state health departments and universities participating in the Emerging Infections Program Network. ABCs conducts population-based active surveillance, collects isolates, and performs studies of invasive disease caused by *Streptococcus pneumoniae*, group A and group B *Streptococcus*, *Neisseria meningitidis*, and *Haemophilus influenzae* for a population of 17 to 30 million. These pathogens caused an estimated 97,000 invasive cases, resulting in 10,000 deaths in the United States in 1998. Incidence rates of these pathogens are described. During 1998, 25% of invasive pneumococcal infections in ABCs areas were not susceptible to penicillin, and 13.3% were not susceptible to three classes of antibiotics. In 1998, early-onset group B streptococcal disease had declined by 65% over the previous 6 years. More information on ABCs is available at www.cdc.gov/ncidod/dbmd/abcs. ABCs specimens will soon be available to researchers through an archive.

Bacterial infections are prototypical emerging diseases (1), and their recent history challenges the premature view that the battle against infectious diseases had been won. In the last 25 years, disease caused by multidrug-resistant *Streptococcus pneumoniae* became established on several continents, reaching the United States by the 1990s (2-4), and fatal infections caused by *S. pyogenes* (group A *Streptococcus*), a problem of the 19th century (5), have returned in toxic and necrotic forms (6). By the 1970s, group B *Streptococcus* replaced gram-negative bacteria and *Staphylococcus aureus* as the leading cause of sepsis in newborns (7,8). Researchers tackled the public health challenge of developing vaccines to protect children against the major causes of bacterial meningitis: *Haemophilus influenzae* type b, *S. pneumoniae*, and *Neisseria meningitidis* (9,10). A critical step for response to microbial adaptation is establishing a reliable tracking system. We describe active, population-based surveillance for serious bacterial infections that was established by the Centers for Disease Control and Prevention

Address for correspondence: Anne Schuchat, Centers for Disease Control and Prevention, Mailstop C23, 1600 Clifton Rd., Atlanta, GA 30333, USA; fax: 404-639-3970; e-mail: aschuchat@cdc.gov.

(CDC) as part of its response to emerging infectious diseases (11,12).

Active Bacterial Core surveillance (ABCs) was designed to estimate the burden of community-acquired invasive bacterial infections that typically manifest as sepsis and meningitis. The system determines incidence and trends of these diseases in a multistate population and uses molecular and microbiologic methods to characterize the organisms causing infection. As prevention strategies against some pathogens are used routinely (9,13,14), ABCs evaluates their impact and identifies missed opportunities for their application. Established in four states in 1995, ABCs now operates within the eight states of the Emerging Infections Program (EIP) network, representing a population of more than 30 million and ascertaining cases from more than 600 clinical microbiology laboratories. A ninth EIP state, Colorado, initiated ABCs during 2000. ABCs currently focuses on surveillance and special studies related to five pathogens: *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, group A *Streptococcus* (*S. pyogenes),* and group B *Streptococcus* (*S. agalactiae).*

ABCs' predecessor was the active surveillance program for invasive bacterial diseases established in 1988 (also sponsored by CDC), which evaluated the efficacy of *H. influenzae* type b vaccines in infants (15), identified dietary risk factors for sporadic listeriosis (16,17), and compared the cost-effectiveness of strategies for preventing group B streptococcal disease in newborns (18). ABCs has expanded the scope of targeted conditions to address additional emerging infections such as necrotizing fasciitis (the so-called flesh-eating disease) and streptococcal toxic-shock syndrome, both severe manifestations of disease caused by group A *Streptococcus.* ABCs also now monitors the emergence of drug resistance in the community-acquired pathogen *S. pneumoniae*. ABCs is one of three core activities conducted by EIPs; the others are FoodNet (19) and the Unexplained Critical Illness and Death Project (20). This article, a progress report of the first 5 years of the EIP network's ABCs project, identifies easily accessible resources from this system for public health and infectious disease constituencies.

ABCs Methods

In 1999, ABCs was conducted in Connecticut as well as in part or all of the following states: California, Georgia, Maryland, Minnesota, New York, Oregon, and Tennessee (Figure 1). (For certain pathogens, surveillance is conducted statewide in Georgia, Maryland, Minnesota, and Oregon). The total population under surveillance in 1998 ranged from approximately 17.4 million for *S. pneumoniae* to 30.4 million for *N. meningitidis*.

Figure 1. States included in Active Bacterial Core surveillance in 1999. Surveillance for all pathogens was conducted statewide in Connecticut but in selected counties only for some or all pathogens in the other states.

A case is defined as isolation of one of the five pathogens from a usually sterile site (e.g., blood, cerebrospinal fluid, pleural fluid) in a resident of one of the surveillance areas. Detailed methods of case-finding, data collection, and laboratory audits conducted within ABCs have been described (10,21). The key features are active ascertainment of cases by state-based surveillance officers, who make regular contact with microbiology or infection control practitioners in all clinical microbiology laboratories processing sterile site cultures for the surveillance area; collection of isolates of the specified pathogens for laboratory testing by ABCs personnel (Table 1); and semiannual audits of all participating laboratories to identify missed cases. Because the surveillance is population-based and cases identified by audits are included in the final database, ABCs data are used to monitor incidence of these diseases in a large, defined population. With the use of race- and age-adjustment, ABCs data also permit annual projections of the estimated incidence as well as the estimated number of cases and deaths occurring in the entire United States. For national projections, cases with unknown race

Table 1. Laboratory characterization of isolates collected as part of the Active Bacterial Core surveillance program

Pathogen	Test(s)
Group A *Streptococcus*	*emm*- and T-typing for all invasive isolates; antimicrobial susceptibility testing of periodic samples
Group B *Streptococcus*	Serotyping and antimicrobial susceptibility testing of isolates for selected surveillance areas
Haemophilus influenzae	Serotyping of all isolates (a-f); molecular subtyping of isolates as part of special projects
Neisseria meningitidis	Molecular subtyping of isolates in conjunction with vaccine development; antimicrobial resistance on isolates periodically
Streptococcus pneumoniae	Antimicrobial susceptibility testing for all invasive isolates; serotyping on all invasive isolates since January 1, 1998; subtyping of a sample of isolates using genotyping methods

are distributed by area, on the basis of reported race distribution for known cases within eight age categories. U.S. census data for counties under surveillance and natality data on live births are the sources of denominators for incidence calculations; the most recent year's population data available with age and race information at the county level are used for rate calculations.

Core surveillance activities include collecting epidemiologic and clinical data and characterizing isolates in terms of antimicrobial susceptibility, serotype or serogroup, and subtyping. ABCs also conducts special studies that use the surveillance infrastructure but require collection of additional data by chart review, patient interviews, or analysis of ABCs data together with complementary data sources. ABCs uses the following indicators to monitor performance: sensitivity of >90% for active surveillance (based on total cases detected by surveillance and the laboratory audit); collection of >85% of isolates from cases; and enrollment of 90% of eligible participants into special studies.

ABCs is overseen by a steering committee consisting of CDC and state EIP representatives as well as external advisors from the public health, infectious disease, and microbiology fields. These parties convey views from key constituents and annually evaluate the need to add or subtract pathogens for surveillance. In 1999, CDC's National Center for Infectious Diseases awarded $10.7 million through cooperative agreements to eight EIP states; approximately $2.5 million (23%) of these funds supported ABCs-related activities.

Results

Surveillance Highlights

In 1998, 6,992 cases of invasive disease caused by the five pathogens were reported from the eight sites. The rates of invasive disease (per 100,000) ranged from 1.0 for *N. meningitidis* to 24.1 for *S. pneumoniae* (Table 2). An estimated 97,000 invasive infections and 10,000 deaths per year in the United States are due to *S. pneumoniae*, group A and B streptococci,

Table 2. Incidence, case-fatality ratio, projected U.S. cases and deaths, and proportion nonsusceptible to penicillin of invasive disease identified in the Active Bacterial Core surveillance (ABCs), 1998

	Group A *Streptococcus*	Group B *Streptococcus*	*Haemophilus influenzae*	*Neisseria meningitidis*	*Streptococcus pneumoniae*
Aggregate incidence[a]	3.8	6.5	1.4	1.0	24.1
Range by area[a]	2.6 - 4.1	4.8 - 8.5	1.1 - 2.3	0.6 - 2.0	20.0-28.9
Case- fatality ratio in ABCs areas	12.2%	9.5 %	13.9%	13.7%	9.3%
Projected U.S. cases	10,200	17,400	3,900	2,500	63,000
Projected U.S. deaths	1,300	1,700	500	400	6,100
Penicillin nonsusceptibility[b]	0	0	- -	1.1%	25.0%

[a]Incidence = cases per 100,000.
[b]Nonsusceptible includes isolates classified as either intermediate or resistant to penicillin. Results reflect testing of group A streptococcal isolates from 1997 (n=183) and group B streptococcal isolates from 1997 and 1998 combined (n=188).

H. influenzae, and *N. meningitidis*. Despite continued availability of effective antimicrobial agents for each pathogen, approximately 1 in 10 cases results in death (Table 2). Substantial geographic variation exists in the incidence of invasive infections caused by each pathogen (Table 2). Among invasive *S. pneumoniae* infections, the proportion caused by drug-resistant organisms was three times higher in some areas than others (4); 8.4% of invasive pneumococci from New York were fully resistant to penicillin (MIC $\geq$2.0), while 25.4% of isolates from Tennessee were penicillin resistant. No penicillin-nonsusceptible (intermediate or resistant) strains of group A or group B *Streptococcus* have been detected to date.

Recent temporal changes are most dramatic for invasive group B streptococcal disease among infants less than 1 week old (i.e., early-onset disease), which declined 65% from 1993 to 1998 (Figure 2), during a period when the incidence of disease in older infants and adults remained stable (22). Data from ABCs provide a reliable standard for evaluating alternative methods for surveillance of drug resistance in *S. pneumoniae*, including sentinel surveillance methods (4) and use of aggregate data from antibiograms from multiple hospitals (23). The recent emergence of serogroup Y meningococci, demonstrated by ABCs, suggests that vaccine companies should consider incorporating serogroup Y in new meningococcal vaccines. In addition, the diversity in the outer membrane proteins of serogroup B meningococcal strains suggests that vaccines against these proteins may not be efficient means of preventing endemic serogroup B meningococcal disease.

Applied Research

The population-based collections of isolates from ABCs are used to evaluate subtyping methods (24), identify genetic mechanisms of antimicrobial resistance, determine vaccine formulations (25,26), and assess capsular switching among organisms (for vaccines based on capsular types) (27,28). ABCs has identified population-based risk factors for disease in various age groups (Table 3). A case-control study of invasive pneumococcal disease in young children showed that attendance at day care was associated with a substantial attributable risk for disease (29). A similar study of invasive pneumococcal disease in 18- to 64-year-old adults who were not immunocompromised identified active and passive smoking, in addition to household contact with a child in day care, as independent risk factors for disease (30). Models of age- and serogroup- or serotype-specific rates of invasive meningococcal and pneumococcal disease in the ABCs population have compared the potential impact of diverse immunization strategies for meningococcal and combined meningococcal-pneumococcal vaccines on disease prevention (32). The increased risk for pneumonia death occurring several days after illness onset associated with antimicrobial-resistant strains of *S. pneumoniae* was demonstrated by using multistate clinical and epidemiologic data from ABCs (33).

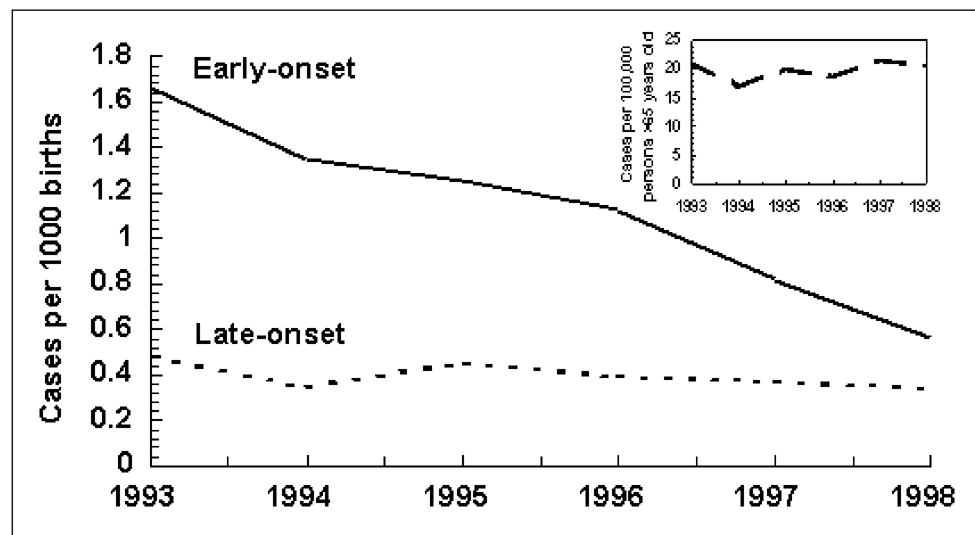


Figure 2. Invasive group B streptococcal disease in infants less than 1 week of age per 1,000 live births and in adults $\geq$65 years of age per 100,000 population, Active Bacterial Core surveillance, 1993-1998 (adapted from ref. 22).

Table 3. Population-based risk factors for invasive disease, identified by Active Bacterial Core surveillance

Pathogen	Age group, other criteria	Factors associated with increased risk by multivariate analysis	References
Streptococcus pneumoniae	<5 years old	Child-care attendance, underlying conditions, lack of breast-feeding, household crowding	29
	18-64 years old, not immuno-compromised	Active or passive smoke exposure, black race, chronic diseases, household contact with child in day care	30
Neisseria meningitidis	All ages	Active or passive smoke exposure, underlying conditions, steroid use, attendance at new school	31
Group B *Streptococcus*	<7 days old	Black race, low birth weight, maternal age <20 years	CDC, unpub. data

Infrastructure

ABCs provides participating state health departments active contact with all acute-care hospitals and reference microbiology laboratories in the surveillance area. This network provides an infrastructure for public health communication and education, as well as a network of key contacts available for response to new or emerging concerns. Periodic surveys of laboratories within ABCs determined the adequacy of methods used to detect group B *Streptococcus* from prenatal screening specimens (34), the computerization of clinical microbiology laboratories and readiness for electronic laboratory-based reporting, and the routine procedures used by ABCs laboratories to detect antimicrobial resistance among *S. pneumoniae*, *S. aureus,* and several other organisms (35). ABCs and other EIP personnel have provided assistance with multistate response efforts to determine the burden of Creutzfeldt-Jakob disease (36) and contributed to efforts to determine the rate of rotavirus vaccine-related intussusception (37). Further, the presence of ABCs personnel in state health departments and academic institutions has strengthened communication links required for accurate reporting and feedback.

Prevention

Since publication of consensus guidelines for the prevention of group B streptococcal disease in newborns, ABCs assessed the implementation of prevention practices and identified opportunities for preventing more cases. ABCs showed that hospital obstetric programs' adoption of policies to prevent group B streptococcal infection increased significantly (38) and that hospitals that had adopted or revised a policy in 1996 had significantly fewer cases in 1997 (39). ABCs is also tracking the characteristics of newborn group B streptococcal cases that continue to occur despite prevention guidelines to determine whether these represent failures of intrapartum antibiotic prophylaxis or failure to offer such prophylaxis to mothers at risk. In several EIPs, pilot prevention programs are in place to identify efficient ways to reduce the incidence of disease caused by ABCs pathogens. These include a multifaceted program to reduce inappropriate antibiotic use in the Baltimore metropolitan area and efforts to promote pneumococcal polysaccharide vaccine in populations at high risk in Rochester, New York; Minneapolis-St. Paul, Minnesota; metropolitan Atlanta, Georgia; and Portland, Oregon. The Connecticut and Minnesota health departments conducted demonstration projects that integrated prevention of group B streptococcal disease into routine perinatal care, building on successes with hepatitis B perinatal prevention programs and contributing to reduction of perinatal HIV transmission (40,41).

Discussion

Nearly 100,000 invasive infections and 10,000 deaths caused by ABCs pathogens occur annually in the United States. Because few states routinely collect data and isolates for all of these infections, ABCs helps monitor disease and evaluate prevention programs at the national level. ABCs has now developed robust estimates of the magnitude of disease and deaths attributable to the five invasive pathogens (Table 2). A number of future priorities have been identified

that take advantage of the careful characterization of isolates associated with invasive infection. Licensure and introduction of a seven-valent conjugate vaccine against *S. pneumoniae* necessitate evaluation of the impact of this new prevention tool on target populations (Table 4). Of particular interest will be evaluating whether indirect effects similar to those seen with the Hib vaccine (42) occur. The large birth cohort under surveillance through ABCs and the longitudinal data on both early-onset cases and hospital policies for disease prevention offer the opportunity to compare the two alternative strategies for group B streptococcal prevention (screening-based vs. risk-based) through two studies during the next few years. ABCs will continue to contribute to tracking progress in Hib elimination, monitor for emergence of other serotypes of *H. influenzae*, and provide data on strain-specific disease (e.g., serotype, serogroup, outer membrane type). Such information is valuable for evaluating new vaccines for group B *Streptococcus* and serogroup B meningococcus. ABCs data will also be used to define clusters of invasive group A streptococcal disease and to model the impact of possible strategies and new formulations of pneumococcal vaccines targeted against pneumococcal pneumonia in adults (Figure 3) as well as vaccines targeted against invasive group A *Streptococcus* syndromes.

The molecular biology revolution and improved understanding of host-pathogen interactions offer great potential to advance knowledge about ABCs bacteria. Emerging antimicrobial resistance and other forms of pathogen adaptation (e.g., capsular switching) lend an urgency to such research. Specimens from invasive disease surveillance represent well-characterized, population-based collections with relevant clinical and demographic information. These provide a valuable resource for basic and applied research focused on issues as varied as new drug and vaccine development, evaluation of mechanisms of virulence and antimicrobial resistance, and genetic research. ABCs is planning to make these strains available to outside researchers and industry through a preserved collection. Such a specimen bank could provide a lasting legacy of the work of hundreds of infection control practitioners, clinical microbiology laboratories, and ABCs surveillance collaborators.

Table 4. Future priorities for Active Bacterial Core surveillance (ABCs) project

1. Define invasive group A *Streptococcus* clusters.
2. Determine effectiveness of screening vs. risk-based prevention strategies for perinatal group B streptococcal disease.
3. Determine feasibility of eliminating invasive disease caused by *Haemophilus influenzae* type b.
4. Quantify culture-negative, polymerase chain reaction-positive meningitis.
5. Measure direct and indirect effects of introducing a seven-valent pneumococcal conjugate vaccine.

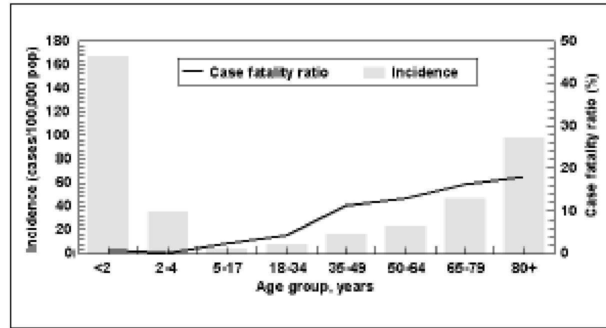


Figure 3. Age-specific incidence (per 100,000) and case-fatality ratio (percent) of invasive pneumococcal disease, Active Bacterial Core surveillance, 1998.

To ensure that ABCs' lessons learned within the EIP network reach other public health constituents, a number of efforts are under way. Additional details of the surveillance system and outreach materials are available at http://www.cdc.gov/ncidod/dbmd/abcs. Other educational materials are available at http://www.cdc.gov/ncidod/dbmd/gbs and at http://www.cdc.gov/ncidod/dbmd/antibioticresistance. For laboratories evaluating new strains of group A *Streptococcus*, genetic sequencing data of all strains described thus far are also available on the web. A similar site for meningococcal isolates is under development.

Conclusions

ABCs is a model of collaboration between public health and academia. The system provides reliable data that can be used to address critical public health concerns, improve understanding of emerging infections, and help prevent the consequences of these infections. While the past 5 years have helped quantify the magnitude of disease caused by these pathogens and document increasing antibiotic resistance in some of them, the future provides several challenges. To remain a vital component

in the nation's efforts to prevent and control emerging infectious diseases, ABCs will need to incorporate surveillance and research tools of the 21st century, including electronic laboratory-based reporting, genotyping of pathogens, and improved communication to promote behavioral change and adoption of practice guidelines.

Acknowledgments

We thank the National Center for Infectious Diseases Emerging Infections Program and infection control practitioners and clinical microbiologists who collaborate on ABCs. We also thank the following additional members of the ABCs team: California: Duc Vugia, Gretchen Rothrock, Pam Daily, Lisa Gelling; Connecticut: James Hadler, Matt Cartter, Pat Mshar, Craig Morin, Aaron Roome, Heather Linardos; Georgia: Paul Blake, David Stephens, Kathryn Arnold, Wendy Baughman, Katherine McCombs, Sabrina Burden, Patricia Martell-Cleary, Mathew Sattah; Maryland: Margaret Pass, Diane Dwyer; Minnesota: Ruth Lynfield, Catherine Lexau, Jean Rainbow, Karen White, Lori Triden, Brenda Sayler; New York: Perry Smith, Shelly Zansky, Barbara Damaske, Nancy Bennett, Glenda Smith, Nellie Dumas, Brian Sauders, Hwa Gan Chang; Oregon: Paul Cieslak, Linda Duke; Tennessee: Allen Craig, William Schaffner, Brenda Barnes, Carolyn Gilmore; CDC: Katherine Robinson, Chris Van Beneden, Cynthia Whitney, Nancy Rosenstein, Carolyn Wright, Kathleen Shutt, Melissa Berkowitz, Falgunee Parekh, Richard Facklam, Bernard Beall, John Elliott, and Tanja Popovic.

Dr. Schuchat is chief of the Respiratory Diseases Branch at the Centers for Disease Control and Prevention. Her research interests include group B streptococcal disease, evaluation of vaccines against meningitis and pneumonia, and prevention of perinatal infections.

References

1. Lederberg J, Shope RE, Oaks SCJ. Emerging infections: microbial threats to health in the United States. Washington: National Academy Press; 1992.
2. Centers for Disease Control and Prevention. Defining the public health impact of drug-resistant *Streptococcus pneumoniae*: report of a working group. MMWR Mor Mortal Wkly Rep 1996;45(No.RR-1):1-14.
3. Klugman KP. Pneumococcal resistance to antibiotics. Clin Microbiol Rev 1990;3:171-96.
4. Centers for Disease Control and Prevention. Geographic variation in penicillin resistance in *Streptococcus pneumoniae*—selected sites, United States, 1997. MMWR Mor Mortal Wkly Rep 1999;48:656-61.
5. Charles D, Larsen B. Streptococcal puerperal sepsis and obstetric infections: a historical perspective. Rev Infect Dis 1986;8:411-22.
6. Schwartz B, Facklam RR, Breiman RF. Changing epidemiology of group A streptococcal infection in the USA. Lancet 1990;336:1167-71.
7. Freedman RA, Ingram DL, Gross I, Ehrenkranz PA, Warshaw JB, Baltimore RS. A half century of neonatal sepsis at Yale, 1928 to 1978. Am J Dis Child 1981;135:140-4.
8. McCracken GH. Group B streptococci: the new challenge in neonatal infections. J Pediatr 1973;82:703-6.
9. Centers for Disease Control and Prevention. Recommendations for use of *Haemophilus influenzae* b conjugate vaccines and a combined diphtheria, tetanus, pertussis and *Haemophilus* b vaccine. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Mor Mortal Wkly Rep 1993;42(RR-13):1-15.
10. Schuchat A, Robinson K, Wenger JD, Harrison LH, Farley M, Reingold AL, et al. Bacterial meningitis in the United States in 1995. N Engl J Med 1997;337:970-6.
11. Centers for Disease Control and Prevention. Addressing emerging infectious disease threats: a prevention strategy for the United States. Atlanta: Centers for Disease Control and Prevention; 1994.
12. Centers for Disease Control and Prevention. Preventing emerging infectious diseases: a strategy for the 21st century. Atlanta: Centers for Disease Control and Prevention; 1998.
13. Schuchat A, Whitney C, Zangwill K. Prevention of perinatal group B streptococcal disease: a public health perspective. MMWR Mor Mortal Wkly Rep 1996;45(No. RR-7):1-24.
14. Centers for Disease Control and Prevention. Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Mor Mortal Wkly Rep 1997;46(RR-8):1-24.
15. Wenger JD, Pierce R, Deaver K, Plikaytis BD, Facklam RR, Broome CV. Efficacy of *Haemophilus influenzae* type b polysaccharide-diphtheria toxoid conjugate vaccine in US children aged 18-59 months. Lancet 1991;338:395-8.
16. Schuchat A, Deaver K, Wenger JD, Plikaytis BD, Mascola L, Pinner RW, et al. Role of foods in sporadic listeriosis. I: case-control study of dietary risk factors. JAMA 1992;267:2041-5.
17. Pinner RW, Schuchat A, Swaminathan B, Hayes PS, Deaver KA, Weaver RE, et al. Role of foods in sporadic listeriosis. II: microbiologic and epidemiologic investigation. JAMA 1992;267:2046-50.
18. Mohle-Boetani J, Schuchat A, Plikaytis BD, Smith D, Broome CV. Comparison of prevention strategies for neonatal group B streptococcal infection: a population-based economic analysis. JAMA 1993;270:1442-8.
19. Centers for Disease Control and Prevention. The Foodborne Diseases Active Surveillance Network, 1996. MMWR Mor Mortal Wkly Rep 1997;46:258-61.
20. Perkins BA, Flood JM, Danila R, Holman RC, Reingold AL, Klug LA, et al. Unexplained deaths due to possibly infectious causes in the United States: defining the problem and designing surveillance and laboratory approaches. Emerg Infect Dis 1996;2:47-53.
21. Zangwill KM, Schuchat A, Wenger JD. Group B streptococcal disease in the United States, 1990: report from a multistate active surveillance system. MMWR Mor Mortal Wkly Rep 1992;41(SS-6):25-32.
22. Schrag S, Zywicki S, Farley MM, Reingold AL, Harrison LH, Lefkowitz LB, et al. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis, 1993-1998. N Engl J Med 2000;342:15-20.

23. Chin AE, Hedberg K, Cieslak PR, Cassidy M, Stefonek KR, Fleming DW. Tracking drug-resistant *Streptococcus pneumoniae* in Oregon: an alternative surveillance method. Emerg Infect Dis 1999;5:688-93.
24. Beall B, Facklam R, Thompson T. Sequencing the *emm*-specific PCR products for routine and accurate typing of group A streptococci. J Clin Microbiol 1996;34:953-8.
25. Harrison LH, Dwyer DM, Johnson JA. Emergence of serotype V group B streptococcal infection among infants and adults. J Infect Dis 1995;171:513.
26. Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliott J, Facklam R, et al. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis 1996;173:365-73.
27. Swartley JS, Marfin AA, Edupuganti S, Liu LJ, Cieslak P, Perkins B, et al. Capsule switching of *Neisseria meningitidis*. Proc Natl Acad Sci USA 1997;94:271-6.
28. Gherardi G, Whitney C, Facklam R, Beall B. Major related sets of antibiotic-resistant pneumococci in the United States as determined by pulsed-field gel electrophoresis and *pbp1a-pbp2b-pbp2x*-dhf restriction profiles. J Infect Dis 2000;181:216-29.
29. Levine OS, Farley MM, Harrison LH, Lefkowitz L, McGeer A, Schwartz B. Risk factors for invasive pneumococcal disease in children: a population-based case-control study in North America. Pediatrics 1999;103:e28.
30. Nuorti JP, Butler JC, Farley MM, Harrison LH, McGeer A, Kolczak MS, et al. Cigarette smoking and invasive pneumococcal disease. N Engl J Med 2000;342:681-9.
31. Fischer M, Harrison L, Farley M, Lefkowitz L, McGeer A, Schuchat A, et al. Risk factors for sporadic meningococcal disease in North America. Philadelphia: Infectious Diseases Society of America; 2000.
32. Lingappa J, Zell E, Rosenstein N, Schuchat A, Perkins BA, Active Bacterial Core Surveillance Team. Impact of vaccination strategies using meningococcal conjugate vaccines in the United States. Philadelphia: Infectious Disease Society of America; 1999.
33. Feikin DR, Schuchat A, Kolczak M, Barrett NL, Harrison LH, Lefkowitz L, et al. Mortality from invasive pneumococcal pneumonia in the era of antibiotic resistance, 1995-1997. Am J Public Health 2000;90:223-9.
34. Centers for Disease Control and Prevention. Laboratory practices for prenatal group B streptococcal screening and reporting—Connecticut, Georgia, and Minnesota, 1997-1998. MMWR Mor Mortal Wkly Rep 1999;48:426-8.
35. Centers for Disease Control and Prevention. Laboratory capacity to detect antimicrobial resistance. MMWR Mor Mortal Wkly Rep 2000;48:1167-71.
36. Centers for Disease Control and Prevention. Surveillance for Creutzfeldt-Jakob disease—United States. MMWR Mor Mortal Wkly Rep 1996;45:665-8.
37. Centers for Disease Control and Prevention. Intussusception among recipients of rotavirus vaccine—United States, 1998-1999. MMWR Mor Mortal Wkly Rep 1999;48:577-81.
38. Centers for Disease Control and Prevention. Adoption of hospital policies for prevention of perinatal group B streptococcal disease—United States, 1997. MMWR Mor Mortal Weekly Rep 1998;47:665-70.
39. Factor SH, Whitney CG, Zywicki S, Schuchat A, the ABC Surveillance Team. Effects of hospital policies on the 1996 Group B streptococcal consensus guidelines. Obstet Gynecol 2000;95:377-82.
40. Roome A, Carley K, Melchreit R, Foye G, Hadler J. Testing pregnant women for HIV. A survey of obstetricians and review of patient prenatal/obstetric medical records—Connecticut 1996-1997. Conn Med 1999;63:541.
41. Lynfield R, Rubin M, White K, Schuchat A, Moore K, Osterholm M, et al. Prenatal HIV screening practices in Minnesota. Philadelphia: Infectious Diseases Society of America; 1999.
42. Adams WG, Deaver KA, Cochi SL, Plikaytis BD, Zell ER, Broome CV, et al. Decline of childhood *Haemophilus influenzae* type b disease in the Hib vaccine era. JAMA 1993;269:221-6.

Emerging Chagas Disease: Trophic Network and Cycle of Transmission of *Trypanosoma cruzi* from Palm Trees in the Amazon

Antonio R.L. Teixeira,* Pedro Sadi Monteiro,* José M. Rebelo,† Enrique R. Argañaraz,* Daniela Vieira,* Liana Lauria-Pires,* Rubens Nascimento,* Cássia A. Vexenat,* Antonio R. Silva,† Steven K. Ault,‡ and Jackson M. Costa‡
*University of Brasília, †Federal University of Maranhão, and ‡Pan-American Health Organization/World Health Organization, Brasília, Brazil

A trophic network involving molds, invertebrates, and vertebrates, ancestrally adapted to the palm tree (*Attalaea phalerata)* microhabitat, maintains enzootic *Trypanosoma cruzi* infections in the Amazonian county Paço do Lumiar, state of Maranhão, Brazil. We assessed seropositivity for *T. cruzi* infections in the human population of the county, searched in palm trees for the triatomines that harbor these infections, and gathered demographic, environmental, and socioeconomic data. *Rhodnius pictipes* and *R. neglectus* in palm-tree frond clefts or in houses were infected with *T. cruzi* (57% and 41%, respectively). Human blood was found in 6.8% of *R. pictipes* in houses, and 9 of 10 wild *Didelphis marsupialis* had virulent *T. cruzi* infections. Increasing human population density, rain forest deforestation, and human predation of local fauna are risk factors for human *T. cruzi* infections.

The tropical moist broadleaf forests of Latin America are an important region for conservation of biodiversity (1). In the Amazon Basin (area 8,214,284 km^2), blocks of original habitat are still intact, while some ecoregions[1] are almost completely converted or degraded, allowing major components of biodiversity to steadily erode (1,2). Populations of several endangered wildlife species have declined, and human habitat and land use are considered a threat to most native species and communities.

Palm trees propagate in ecoregions of the Amazon Basin ecosystem amid other vegetation or in enormous palm forests. Of approximately 2,800 palm species worldwide, 387 (13.8%) are native to the basin (3,4). Palm trees have been used to study the evolution of biological diversity and are excellent markers of ecologic fitness in the Amazon Basin (2,4). In addition, these trees may play an important role in the forest ecosystem. Palm trees produce 15 tons of dry organic material per hectare per year (threefold more than other species of trees) and recuperate more rapidly after fire than other forest species. A single native palm tree may serve as shelter and food for diverse fauna (wild mammals, snakes, scorpions, spiders, amphibians, and many species of insects). Palm trees are also an important economic resource for residents of the Amazon region, who collect and sell palm roots, stipe, leaves, fruits, seeds, heart of palm, and inflorescences (4,5). The babassu palm *(Attalaea phalerata)* reaches an average density of 200 trees per hectare in the state of Maranhão, but lower densities were reported in the states of Piaui, Goiás-Tocantins, and Mato Grosso (5), where babassu trees number an estimated 11 x 10^8.

Address for correspondence: Antonio R.L. Teixeira, Chagas' Disease Multidisciplinary Research Laboratory, Faculty of Medicine, University of Brasília. P.O. Box 04536. Postcode 70.919.970, Brasília, DF, Brazil; fax: 55+61 273-4645; e-mail: ateixeir@unb.br.

[1]In this study, a major ecosystem is defined as a set of ecoregions of comparable dynamics, response characteristics to disturbance, species diversity, and conservation needs. An ecoregion is a geographically distinct set of natural communities with similar species, ecologic dynamics, environmental conditions, and ecologic interactions critical for long-term persistence (1).

Not much attention has been given to human health conditions in this ecosystem (6), probably because pathologic conditions in tropical broadleaf forests are difficult to quantify in these isolated, often impoverished communities. Nineteen sylvatic species of triatomines were identified in the Amazon Basin (7-10), six in association with palm tree microhabitats (Table 1). Eleven of these species were infected with *Trypanosoma cruzi* or *T. cruzi*-like flagellates (17,18). None of these triatomine species, with the possible exception of *T. rubrofasciata*, have adapted to human habitats in the Amazon Basin (19). Since 1924, when *T. cruzi* infection in wild squirrel monkeys (*Crisotrix sciureus*) was described (20), sporadic human *T. cruzi* infections have been reported in the basin (21). However, this enzootic protozoan infection received attention only after 1969, when acute cases of human Chagas disease were described in Belém, State of Pará, Brazil (22,23). Further evidence shows that *T. cruzi* infections are endemic in the Amazon Basin (12-15,21-28) (Figures 1 and 2).

Table 1. Reservoir hosts, triatomines and palm trees participating in the life cycle of transmission of *Trypanosoma cruzi* in the Amazon Basin

Mammal hosts of *T. cruzi*[a]
Primata
Marsupialia
Edentata
Rodentia
Carnivora
Artiodactyla
Chiroptera
Triatomines
Belminus herreri
Cavernicola lenti
C. pilosa[b]
Eratyrus mucronatus[b]
Microtriatoma trinidadensis[b]
Panstrongylus geniculatus[b]
P. rufotuberculatus[b]
P. lignarius[b]
P. arthruri
Rhodnius brethesi
R. nasutus
R. neglectus[b]
R. paraensis[b]
R. pictipes[b]
R. prolixus[b]
R. robustus[b]
Triatoma maculata
T. rubrofasciata
T. rubrovaria
Palm trees
Acrocomia aculeata
A. sclerocarpa[c]
Astrocaryum aculeatum
Attalaea phalerata[c]
A. vulgare
Bactris gasipaes
Euterpe oleracea[c]
E. precatoria
Leopoldina piassaba
Mauritia flexuosa[c]
Maximiliana elegans[c]
M. regia
Oenocarpus bacaba
O. bataua
O. mapora
Phytelephas macrocarpa
Scheelea martiana
Sheelea sp.[c]

[a]>100 mammal wildlife species are reservoirs of *T. cruzi* (11).
[b]Triatomine species found with *T. cruzi* infection (12-14).
[c]Palm species with triatomines infected with *T. cruzi* (13,15,16).

Spellerberg and Hardes (29) describe the major threats to rain forest conservation as shifting agriculture, cattle ranching, logging, and industrialization (mining, hydroelectric dams), to which we add land colonization. We

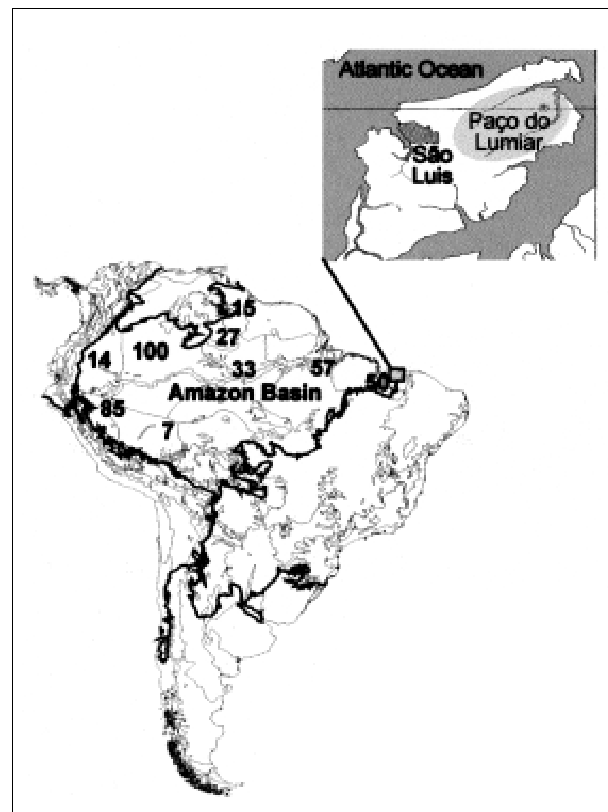


Figure 1. Human cases of acute human *Trypanosoma cruzi* infections in the Amazon Basin (19-28). French Guiana, 15; Colombia, 100; Ecuador, 14; Peru, 85; and Brazilian States: Amapá, 27; Acre, 7; Amazonas, 33; Pará, 57; and Maranhão, 50 cases. Insert shows Paço do Lumiar county in the island São Luis, State of Maranhão, an ecoregion vulnerable to human predation, where acute *T. cruzi* infections have been identified.

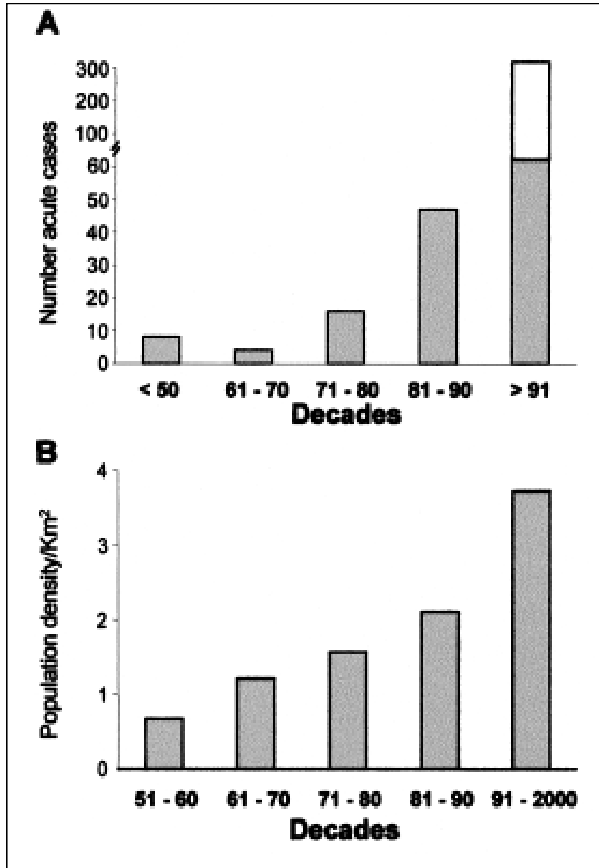


Figure 2. Human population growth and acute *T. cruzi* infections in the Brazilian Amazon Basin (19-28). In A, population density increased 2.5-fold in the last three decades; in B, acute *T. cruzi* infections increased several-fold in the same timespan. ☐ = break in scale.

hypothesized that certain effects of human economic activity (population growth, land use, deforestation, lack of environmental education programs, and human predation on mammals and birds) in a defined ecoregion of this major ecosystem may therefore pose a risk for outbreaks of acute Chagas disease. We evaluated these risk factors in the Tocantins River moist forest ecoregion (1), which is considered vulnerable to human intervention. We conducted a pilot study to assess seropositivity for *T. cruzi* infections in the human population of Paço do Lumiar County, state of Maranhão, Brazil, and to search for triatomines that harbor these infections in palm trees. We also gathered and analyzed demographic, environmental, and socioeconomic data.

Methods

The field study was carried out in Paço do Lumiar county (population 55,000), 20 km from São Luiz, capital of the state of Maranhão. The county is part of the Tocantins moist forest ecoregion (1). We worked in 15 villages, separated by partly deforested argilaceous pathways with scattered houses, where mud-walled, thatch-roofed houses are usually located beneath or beside large palm trees. The surroundings consist of shady, partly deforested areas, where dogs, cats, chickens, pigs, cows, and horses live; no clear delineation separates peridomestic areas from the dense rain forest habitat of wild animals. The county's economy depends on subsistence agriculture and fishing. Raising domestic animals, producing manioc root flour and grains, and harvesting greens and fruits necessitate clearing areas of forest.

We conducted a serologic survey to assess the prevalence of *T. cruzi* infection in 25,451 county residents >1 and <75 years of age (72% were ≤30 years of age) in these 15 villages with <1,200 houses. Fingerprick blood samples from study participants were collected onto Whatman (Clifton, NJ) 1-mm filter paper for seropositivity assessment. After air-drying at room temperature, each set of 10 blood samples was sealed in clean plastic wrap and kept dry in an ice box during the day of collection. The blood samples were then stored frozen until analysis. At the laboratory, filter paper blood samples were punched out and eluted in 100 µL of phosphate-buffered saline (PBS), pH 7.4, as described (30). The test was standardized for obtaining 5 µL of blood in 1 cm^2 of the filter paper, and serum proteins were eluted in 100 µL of PBS, pH 7.4, yielded a 1:20 final dilution for screening seropositivity. For quality control, 10% of the samples were analyzed by a second examiner.

Trapping Triatomines

The strategy for trapping sylvatic triatomine bugs derived from published work (12,17), as well as observations by local residents that triatomines attracted by light fly from palm trees to houses at night. A night visit to one house resulted in capturing two triatomines on the wall near a light bulb; both these specimens had protozoan flagellates in the intestinal contents.

Researchers and field workers spoke to residents at clubs or social organizations. Dried triatomines were displayed, and community leaders requested that triatomines in houses be captured and stored (in a 5x3-cm translucent plastic container with holes in the cap). This strategy proved efficient for collecting triatomines in the rainy season, when they invaded the houses.

We systematically dissected 23 palm trees (*A. phalerata*) in backyards in five villages of the county. Each tree was cut into segments: stipe, crown shaft, fronds, petiole, and leaves. Each segment was carefully searched for insects, mammals, bird nests, and animal vestiges.

During microhabitat dissection, we captured 67 nymphs and 95 adults of three species of triatomines. The precipitin test was used to type blood in the intestinal contents of 44 adult male and female triatomines. The test consisted of two-dimensional immunodiffusion of blood in the insects' intestinal fluid against taxon-specific antisera (31,32).

Trapping and Identifying Wild Animals

The rich bird fauna in the research area included *Aratinga jandaia, Buzeo magnirostris, Colombina passerine, Coragyps atratus, Crotophaga ani, Guira guira, Otus choliba, Pitangus sulphuratus, Turdus fumigatus,* and *Tyranus muscivora*. We did not capture birds because they are refractory to *T. cruzi* infections. However, we captured sylvatic mammals (*Didelphis marsupialis*) near houses in the study area. These ancient marsupials eat palm-tree fruits and rest and nest in the clefts between the stipe and their fronds. They leave these hiding places at night to search for fruits, chicken eggs, baby chicks, and food scraps. Using nylon net or wooden box-traps baited with mango and banana, we captured 12 adult *D. marsupialis* but were unable to trap *Caluromys* sp. seen in the palm trees around the study area.

Biologic Characterization of Kinetoplastid Flagellates

Parasitic protozoa in the feces of insect vectors of Chagas disease infections or in blood agar broth were demonstrated directly by light microscopy. Flagellate protozoan infections in *D. marsupialis* were detected by xenodiagnosis or hemoculture (33). For xenodiagnosis, 20 first-instar uninfected nymphs of *Dipetalogaster maximus* took a blood meal from each adult *D. marsupialis* captured in the field. Thirty days later, the feces of the triatomines were examined by microscopy for flagellates. Any parasitic flagellates in the feces of triatomines or in hemocultures were subjected to passage in weanling mice. This procedure consisted of intraperitoneal injection of a saline dilution of the metacyclic flagellates into mice. Two weeks later, trypomastigote forms of the parasite were identified in the blood of the mice, then 100 µL of infected blood was seeded in blood-agar slants, the supernatant of which yielded parasitic forms, which were used for mass production in nutrient-rich liver infusion tryptose medium. One isolate from *R. pictipes* (*Rp*1) and three isolates from *D. marsupialis (Dm*1, *Dm*2, and *Dm*3) were characterized.

Phenotypic and Genotypic Characterization of *T. cruzi*-like Isolates

Specific antibodies in sera from Chagas disease patients with parasitologically confirmed *T. cruzi* infection were used as phenotypic markers for the counterpart herein called *T. cruzi*-like parasitic infection. Binding of antibodies to epimastigote forms grown in liver infusion tryptose (LIT) medium and to amastigote forms in sections of murine tissues was detected by indirect immunofluorescence assay (30,34). Epimastigote and amastigote forms of the archetype Berenice stock of *T. cruzi* were used as positive controls. For negative controls, parasitic forms from both sources were treated with sera of *T. cruzi* antibody-negative persons.

We extracted DNA of parasitic forms from each of three flagellate protozoa derived from *D. marsupialis* and one isolate of *R. pictipes.* The epimastigote forms grown in LIT were used for extraction of nuclear and kinetoplast DNA, essentially as described (35). DNA samples were analyzed by polymerase chain reaction (PCR) with specific primers for the constant regions of minicircles of kDNA and for highly repetitive sequences of nuclear DNA of *T. cruzi*, as described (35-37). In addition, we used the *r*DNA nested set of primers D71/72A, which can amplify sequences of 125 and 110 base pairs (bp), respectively, from type II or I parasites (38-41). The reactions were run in parallel with 100 pg of protozoan flagellates *Rp1, Dm1, Dm2,* and *Dm3*. As positive controls, we used 100 pg of DNA from *T. cruzi* Berenice (Type 1) and *Dm28* (Type 2). Negative controls were 100 pg of DNA from *Leishmania braziliensis* (42) and *T. rangeli* (43,44).

Formalin-killed epimastigote forms of *T. cruzi*-like flagellates from *Rp*1, *Dm*1, *Dm*2, *Dm*3, and *Dm*4 and the Berenice stock of *T. cruzi* were used. The probes consisted of a nuclear DNA sequence PCR amplified with primer sets TcZ1/2 (37). The probe was labeled with biotin

according to the manufacturer's protocol. Cells fixed in glass slides were hybridized with selected DNA probes and stained with fluorescein-labeled streptavidin (Sigma Chemical Co., St. Louis, MO).

Each of 10 *D. marsupialis* trapped in the wild and BALB/c mice receiving *T. cruzi*-like parasites isolated from triatomines were subjected to histopathologic study. The animals were euthanized, and tissue samples from organs and tissues were fixed in 10% formalin. Three representative sections of skeletal muscles, heart, esophagus, small and large intestine, liver, kidney, spleen, and lung were stained with hematoxylin and eosin for examination by microscopy. An average of six sections from the scent glands of the marsupials was taken for histopathologic study.

Results

By indirect immunofluorescence test for anti-*T. cruzi* antibodies in human blood collected on filter paper (30), 212 (0.83%) of persons tested had specific antibodies for *T. cruzi* infections (Figure 3). Positive serologic results in young populations indicate recent transmission and acute infection. Forty-six children ≤10 years of age (0.18% of the total study population) were antibody-positive for *T. cruzi* and were considered acutely infected.

Our results, which show seroprevalence of *T. cruzi* infections in the absence of hematophagous bugs or their vestiges (excreta and molted skins) in houses, prompted us to search for triatomines in the ecosystem where the population was infected or continues to be at risk. The strategy for capture of triatomines consisted in surveillance of houses by residents (household members captured bugs in the house and placed them in plastic containers) or in dissection of palm trees in backyards of houses (16,45-49). This householder-assisted surveillance and capture method yielded 52 triatomine bugs (36 *R. pictipes* and 16 *R. neglectus*). Triatomine excreta and molted skins in these houses were neither reported by inhabitants nor detected by field workers. Adult triatomines were captured in houses only during the rainy season. We also captured 133 triatomines in 23 palm trees cut down in backyards in five villages. Careful dissection of these trees allowed detection of different developmental stages of triatomines in clefts of palm frond-sheets (Figure 4, Tables 2-4).

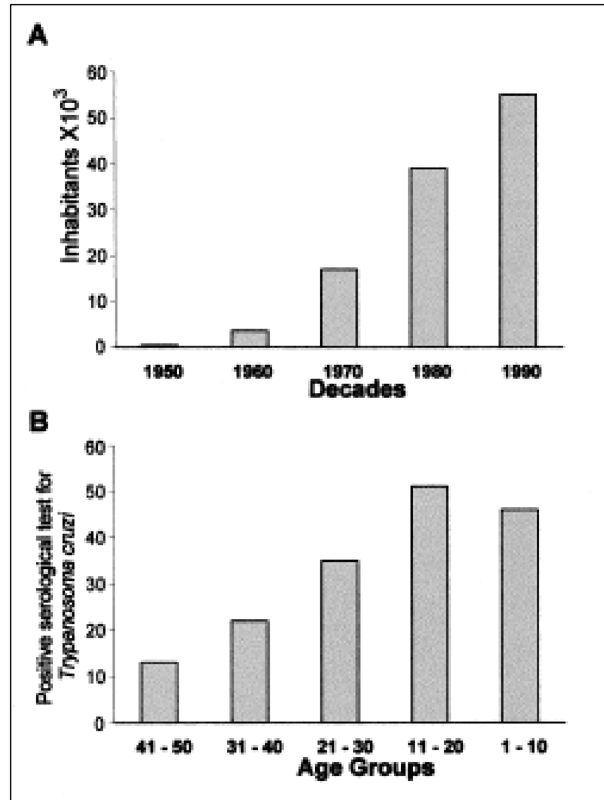


Figure 3. Human population growth and acute *Trypanosoma cruzi* infections in the Amazonian county Paço do Lumiar (19-28). In A, population density increased approximately fourfold in the last three decades. In B, autochtonous acute *T. cruzi* infections increased fourfold in the same timespan (comparing percentages of seropositivity in 41- to 50-year-olds vs. 11- to 20-year-olds), affecting younger age groups.

Figure 4. Palm tree (*Attalaea phalerata*) frond-sheet microsystem and cycle of transmission of *Trypanosoma cruzi.* This natural dwelling of triatomine species and top predators (*Didelphis marsupialis*, circle) consists of clefts formed by the insertion of frond sheets into the stipe of babassu palm.

Remains of animal species (e.g., nests, hair, feathers) on which triatomine bugs prey were identified in palm trees in backyards in five villages. Twice when a tree was cut down, adult opossums (*Didelphis*, Figure 4) ran out of the fronds into the forest. Nests of marsupials and birds were easily detected on dissection of palm fronds and crowns. In addition to opossums and birds, we identified molds and captured and identified different species of various taxa of invertebrate and vertebrate animals (50-60) in the 23 palm trees (Table 5, Figure 5). Molds were found in stipes, fronds, and crowns, and insects in roots, stipes, inflorescence, fruits, fronds, crowns, and leaves. The clefts formed by frond sheets were particularly rich in Amphibia, Arachnida, and Hemiptera. Triatomines were detected at the bottom of clefts where marsupials built their nests. Bird nests were found in the fronds and crowns where abundant species of insects were available for predation.

Scarcity of blood flagellates in marsupials precluded detection by direct microscopy. However, the metacyclic flagellates recovered by xenodiagnosis were subinoculated in weanling mice. Two weeks after injection, trypomastigote forms of the parasitic protozoan morphologically indistinguishable from *T. cruzi* were detected in blood of the mice. To define and further characterize these isolates, we used phenotypic and genotypic molecular characterizations. In the first group, antibodies in sera of chronic Chagas disease patients reacted indistinctly with antigenic determinants in the surface of *T. cruzi* Berenice and with isolates *Dm*1, *Dm*2, and *Dm*3 from *D. marsupialis* and with *Rp1* from *R. pictipes*.

Genotypic kDNA and nuclear DNA (nDNA) markers were used to genetically characterize these wild flagellate protozoan isolates. PCR amplification of template DNA from each of these *T. cruzi* isolates showed that kDNA primers S35/36 (35-37) amplified *T. cruzi*, a laboratory standard for virulent *T. cruzi*. In addition, when we used PCR with mini-exon intergenic spacer primers TC/TC1/TC2 (38) and *r*DNA primers D71/72 (39-41), amplification resulted in the same bands, using template DNA from wild *T. cruzi Dm 28c*, *Dm*1, *Dm*2, *Dm*3, and *Rp*1 and from Berenice *T. cruzi* isolated from a patient with acute Chagas disease (Figure 6). These molecular features allowed classification of wild isolates of

Table 2. Triatomine bugs infected with *Trypanosoma cruzi* in palm trees and houses, Paço do Lumiar, Maranhão, Brazil*

Triatomines	*Rhodnius pictipes*	*R. neglectus*	*Panstrongylus lignarius*	Total (%)
		Palm trees		
No. captured	89	33	11	133
% infected	68	39	27	57
		Houses		
No. captured	36	16	-	52
% infected	28	31	-	41

*The triatomines nymphs and adults were captured either by careful dissection of palm trees or surveillance of houses (12). Flagellate forms in the feces of the Triatomines were detected by microscopy and further identified as *T. cruzi* (see text).

Table 3. Developmental stages of *Rhodnius pictipes, R. neglectus,* and *Panstrongylus lignarius* found in houses and palm trees *Attalaea phalerata**

Reduviid species	*R. pictipes*	*R. neglectus*	*P. lignarius*
		Nymphs	
1st instar	4	3	1
2nd instar	14	-	-
3rd instar	9	5	1
4th instar	6	3	1
5th instar	14	4	2
		Adults	
Male	27	10	3
Female	29	13	3
Total	103	38	11

*No nymphs were found in houses.

Table 4. Type of blood in triatomine bugs captured in palm trees and houses

Triatomine	Total	Bird (%)	Bird/ Didelphis (%)	Didelphis (%)	Rodent (%)	Didelphis/ Rodent (%)	Human (%)	Canine/ Equine (%)
Rhodnius pictipes	28	26.6	26.6	26.6	13.4	6.8	6.8	-
R. neglectus	10	40	-	40	-	-		20
Panstrongylus lignarius	6	100	-	-	-	-		-
%		36.6	18.2	27.3	9.1	4.5	4.5	4.5

*The blood type in the gut of triatomine bugs was identified by the agarose gel precipitin test with taxon-specific antiserum (31,32).

Table 5. Trophic network in randomly selected palm trees of an Amazonian county*

Network	Trophic level†	Palm tree localization	Local given names
Metaphyta:			
Attalaea phalerata	1	Rainforest	Babaçu
Mold: (50, 51)			
Meliolaceae	2	Stipe & crown shaft	Bolor
Meliola acristae			
Catabotrydaceae			
Catobotrys decidium			
Metazoa:			
Insecta (52, 53)			
Coleoptera			
Curculionidae			
Homalinotus coriaceus			
Rhynchophorus palmarum			
R. barbirosstris			
Amerrhynus inca	3	Stipe, fronds & crown	Besouro
Chrysomelidae	3	Crown	Barata do coqueiro
Coraliomela brunea			
Mecistomela marginata			
Scarabaeidae	3	Roots	Rola bosta, broca raiz
Strategus aloeus			
Bruchidae	3	fronds	Broca, bicho do côco
Pachimerus nucleorum			
Homoptera			
Asphidae	3	Crown	Pulgão
Cerataphis lataniae			
Diaspididae	3	Crown	Cochonilha coqueiro
Aspidiotus destructor			
Lepidoptera			
Brassolidae	3	Crown	Borboleta
Brasssolis sophorae			
Brassopholis astyra astyra			
Hymenoptera			
Formicidae			
Acromyrmex histrix			
A. landolti			
A. laticeps migrosetosus	2 & 3	Roots, stipe & fronds	Formiga
A. nobilis			
A. lundi carli			
Hemiptera			
Reduvidae (6, 7, 10, 17)	4	fronds & crown	Barbeiro
Rhodnius pictipes			
R. neglectus			
Panstrongylus lignarus			
Aracnidea, Araneae (38)	4		
Theraphosidae		fronds	Aranha
Amphibia	4	fronds	Perereca
Scinax sp			
Aves (56)	5	fronds & crown	
Aratinga jandaia			Jandaia
Buzeo magnirostris			Gavião
Colombina passerine			Rolinha
Coragyps atratus			Urubú
Crotophaga ani			Anu
Guira-guira			Anu branco
Otus choliba			Caburé
Pizangus sulphuratus			Bem-te-ví
Turdus fumigatus			Sabiá
Tyranus muscivora			Tesourinha
Marsupialia (54-59)	5	fronds	
Didelphis marsupialis			Mucura
Caluromys sp.			Cuíca

*Palm trees were randomly selected from five different localities (backyards) in the Paço do Lumiar county.
†Trophic levels in microhabitat following the flux of energy: 1) Palm tree, predated by molds, insecta, birds and mammals; 2) molds, predated by Formicinae and Ponarinae; 3) Insects, predated by arachnids, amphibians and birds; (4) Arachnids and amphibians, predated by birds; 5) Birds and mammals, predated by hematophagus insects and man (50-60).

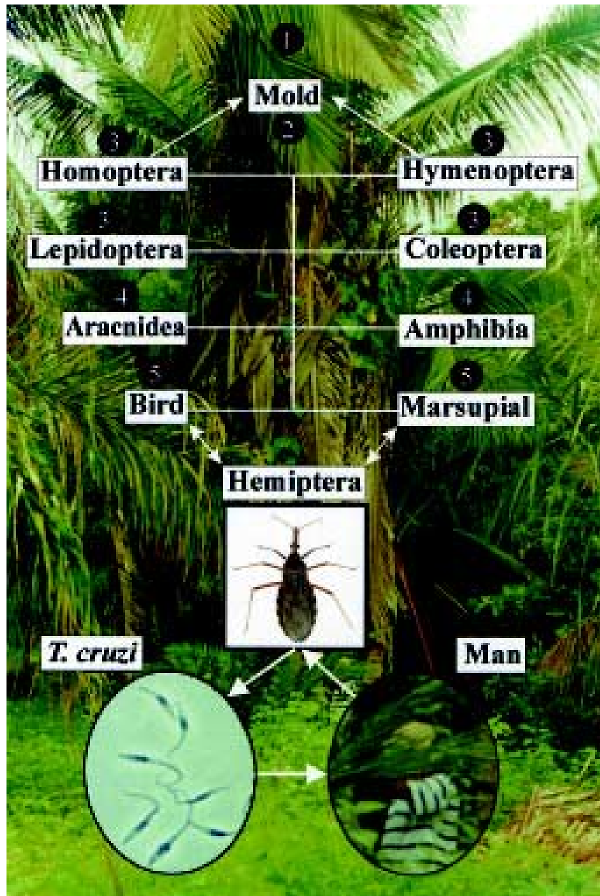


Figure 5. The informal economy: man carrying a babassu palm frond to be sold as soft thatch. A single palm tree enclosing five trophic levels associated with the *Rhodnius pictipes* (Hemiptera: Reduviidae) transmission of *T. cruzi* in the babassu microsystem.

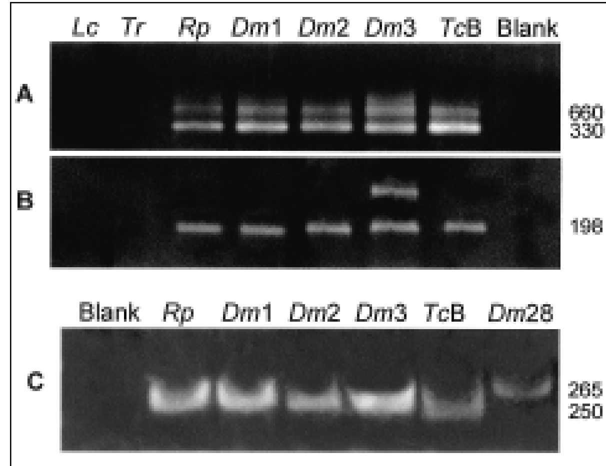


Figure 6. Genotypic characterization of wild-type flagellates by PCR amplification with rDNA and mini-exon specific primers derived from *Trypanosoma cruzi*. A, template DNAs amplified with mini-exon intergenic spacer primers (38): Blank, negative control; Tcb, archetypic type II *T. cruzi* Berenice; *Rp1*, *Dm1*, *Dm2,* and *Dm3*, flagellates isolated from *Rhodnius pictipes* and from *Didelphis marsupialis*; *Dm28*, standard type I, sylvatic *T. cruzi* isolate. B, same template DNAs amplified with rDNA primers (39-41). Tcb yielded typical 300-bp band of type II lineage, whereas *Rp1*, *Dm1*, *Dm2,* and *Dm3* and *Dm28* yielded a 350-bp band of type I *T. cruzi* lineage, with mini-exon spacer primers. In addition, Tcb yielded a typical 125-bp band of type II, whereas the sylvatic *T. cruzi* isolates yielded a 110-bp band of type I, with rDNA primers. These findings confirm sylvatic *Rp1*, *Dm1*, *Dm2*, and *Dm3* as *T. cruzi*.

T. cruzi as phylogenetic type I, while Berenice was *T. cruzi* II (11,38-41). These results were further confirmed by in situ hybridization of wild *T. cruzi* with a biotinilated 198-bp sequence derived from Berenice template DNA (Figure 7), which was amplified with specific nDNA primers Tcz1/2 (37).

Conclusions

Several authors (7,8,17-19,12) described *T. cruzi*-like flagellate protozoans (instead of *T. cruzi*) in the blood of various classes of mammals and in the feces of triatomine species from the Amazon Basin. We detected blood flagellates in 9 of 10 Didelphidae captured in backyards of houses in Paço do Lumiar county. In addition, we isolated flagellates from feces of *R. pictipes*, which showed the same morphologic features described for *T. cruzi*. In biological characterizations, these flagellates induced low-level parasitemia in laboratory mice. However, histopathologic lesions in marsupials and laboratory mice were similar to those described in Chagas disease patients. Furthermore, nuclear DNA markers displayed all features of the *T. cruzi* standards Berenice and *Dm* 28c *T. cruzi*, which differed from those shown by *T. rangeli* and *L. braziliensis*. These data confirm the parasitic flagellates present in triatomines and mammals in this ecoregion as *T. cruzi*.

Birds are refractory to *T. cruzi* infections (33), but several authors (54-60) describe marsupials as important wildlife reservoirs of this parasitic protozoon. We used traps with fruit baits to capture 12 marsupials, which underwent karyotyping and parasitologic and pathologic examinations. The karyotyping confirmed these mammals as *D. marsupialis*. Nine of 10 trapped marsupials had protozoan blood flagellates isolated by xenodiagnosis or hemoculture. Histopathologic study of heart sections from

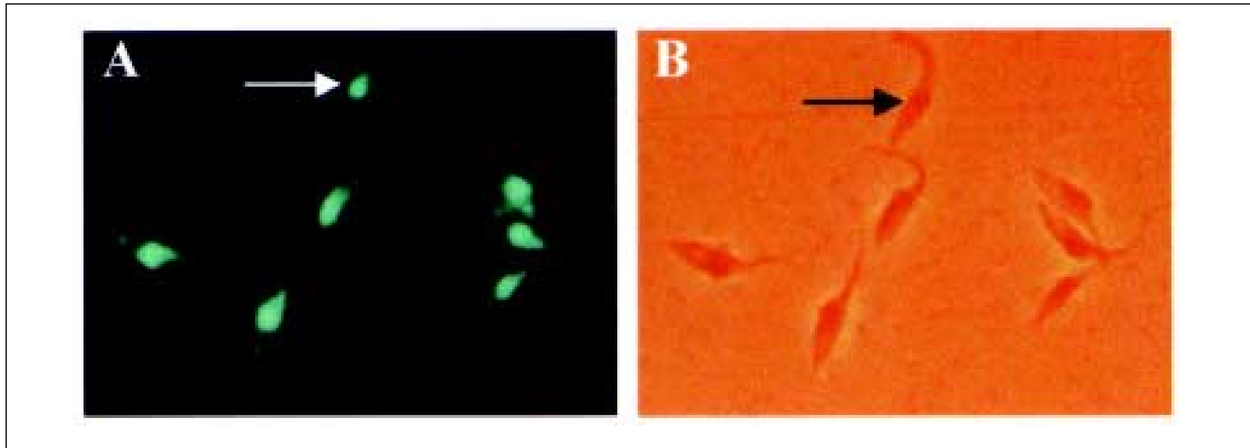


Figure 7. In situ hybridization of *Rhodnius pictipes*-derived flagellates with an archetypic Berenice *Trypanosoma cruzi*-specific probe. The byotinylated 198-bp sequence amplified with Tcz1/2 nuclear DNA probe (37) was revealed with fluorescein-conjugated streptavidin. This genotypic marker, representing 12.5% of total parasitic nuclear DNA, also confirms this sylvatic isolate as *T. cruzi*.

these infected animals showed typical myocarditis, characterized by mononuclear cell infiltrates and target cell lysis. Inflammatory infiltrates were seen in skeletal muscles, esophagus, and small and large intestines. Histopathologic study of representative tissue sections taken from the only opossum that did not have the parasite detected showed no histopathologic alterations (data not shown).

American trypanosomiasis has been considered an ancient zoonosis in which insect vectors and mammal hosts sympatrically occupy vast areas of South America (33). Wild *T. cruzi* infections and the bug vectors are syntopically adapted to mammalian host habitats under natural equilibrium. *T. cruzi* infections and human Chagas disease occur over a large geographic area, limited by parallels 42° N to 42°S (33). The Tocantins moist forest, much of which is subject to severe disturbance of the environmental equilibrium, provided conditions for disease outbreaks (61-64). The growing human populations encroaching on that natural ecoregion are fed upon by the triatomine vector *R. pictipes,* and cases of acute *T. cruzi* infections in humans have exponentially increased in the last three decades.

In this study, we describe a trophic network of five levels comprising different species dwelling in palm tree microhabitats. A single class of top predator mammal (Didelphidae) was found in the study area. The absence of other taxa of top wild predators upon which bugs feed may contribute to peridomiciliar and domiciliar invasion during the wet season. This observation contrasts with earlier descriptions of seven families of mammals, belonging to Primates, Edentates, Marsupials, Carnivores, Rodents, and Chiroptera classes (10,55,57), which were hosts for triatomines in relatively undisturbed ecoregions. Elimination of a single class of invertebrate or vertebrate animals in a trophic network may be a major risk factor leading to more triatomine species entering houses and initiating a new cycle of transmission of *T. cruzi* infection.

In our study, a child $\leq$10 years of age with a positive immunofluorescence test (see methods) was considered a host of acute *T. cruzi* infection. Considering the age-specific prevalence of *T. cruzi* infections in adults (30) and the fact that for each acute case that is clinically identified an estimated 20 to 100 others are unrecognized (34), autochthonous human Chagas disease in the Amazon Basin may reach 7,860 to 39,300 cases. The latter figure is consistent with serologic evidence of *T. cruzi* infection in the Brazilian Amazon region presented in the national report on Chagas disease (65). The characteristics of transmission of infections described here do not indicate a need for insecticide spraying in the Amazon region, for the cycle of transmission of *T. cruzi* is deeply embedded in a natural trophic network comprising wild animals belonging to several classes and trophic levels.

Risk factors associated with the possibility of emergence of endemic Chagas disease in the

Amazon Basin have been described (10,12,17,19,25,54,55,62-64). First, the broadleaf moist rain forest ecosystem may be invaded by triatomine species (*T. infestans* and *T. rubrofasciata*), which are considered completely adapted to human domiciles, or by other triatomines (*P. megistus* and *T. brasiliensis*, *T. pseudomaculata*, and *T. sordida*), which can be found in different ecosystems but frequently enter and colonize houses (64). Second, several Amazon Basin triatomine species (*R. pictipes, R. prolixus, R. neglectus, R. nausutus, T. vitticeps, T. rubrovaria* and others) can adapt to human dwellings, where they could become important vectors of the *T. cruzi* infections (63-69). We found no vestiges of triatomine colonization in houses or their surroundings in our study area. We hypothesize that starving adult *R. pictipes* and *R. neglectus* may leave their natural shelters at night to feed on human hosts, probably attracted by light in the houses.

Factors associated with triatomines flying from palm tree to houses need to be clarified. The scarcity of birds and mammals during the wet season may be an important factor associated with anthropic predation and the presence of *T. cruzi*-infected insects in houses in the rainy season. Domiciliation of triatomines may not be required for an increasing endemicity of Chagas disease in the Amazon Basin (13,14,19). For example, sylvatic *Rhodnius brethesi* recently bit people harvesting palm fibers in Barcelos in the northwestern part of the State of Amazonas, 490 km upriver from Manaus, leading to an acute case of Chagas disease (13). Blood samples from residents of this locality tested by immunofluorescence assay had 12.5% positivity for anti-*T. cruzi* antibodies (70). These observations suggest that to prevent transmission of *T. cruzi* infections to humans in the Amazon Basin, new strategies are needed, which will not necessarily be similar to those used in controlling endemic Chagas disease in other ecosystems, such as the Cerrado and Caatinga ecosystems in Brazil (1,63,66,67,71).

The risk for endemic Chagas disease in the Amazon Basin appears related to deforestation and new population settlements, shifting cultivation, and rapid human colonization of the vectors' natural ecotope in the last three or more decades, as well as to lack of multiple blood sources (hosts) for the vectors in the rainy season. Amazon Basin periurban and urban areas where acute cases of Chagas disease have been reported did not show signs of colonization of houses with triatomine bugs. Instead, transmission of sylvatic *T. cruzi* to humans has been associated with sylvatic species (*R. pictipes*, *R. neglectus, P. lignarius*), which fly from palm trees to houses. New prevention and control strategies should take into consideration risk factors leading to endemicity of the disease in the Amazon. An entomologic and epidemiologic system for surveillance of Chagas disease in Amazonia has been suggested (68,69).

Alternatively, the enormous task of controlling emerging Chagas disease in the Amazon Basin should rely initially on an information, education, and communication program, which encourages control measures by the householder (e.g., use of screens, bed nets, insecticide-treated fabrics, and vegetation management) (69-73). Such a program for prevention of contact with triatomines should be conducted directly in communities, elementary schools, and churches and social clubs, reinforced by social marketing and mass media communications. Further studies are also needed for identifying new and integrated (chemical and nonchemical) strategies required for controlling *T. cruzi* vectors in the Amazon Basin, which may not necessarily be similar to those already shown to be partially effective in controlling the domestic vectors of endemic Chagas disease in other ecosystems in the Americas.

Acknowledgments

We thank the National Laboratory for Taxonomy of Triatomines, Fundação Instituto Oswaldo Cruz, Rio de Janeiro, and the Chagas Disease Multidisciplinary Research Laboratory, University of Brasília, for performing the precipitin test.

Dr. Teixeira is professor of pathology at the medical school of the University of Brasilia. His research interests focus on endemic infections by kinetoplastic flagellates and the pathogeneses of Chagas disease and cutaneous and visceral leishmaniasis.

References

1. Dinerstein E, Olson DM, Graham DJ, Webster AL, Primm SA, Bookbinder MP, et al. A conservation assessment of the terrestrial ecoregions of Latin America and the Caribbean. Ed. Washington: The World Wildlife Fund/The World Bank; 1995.
2. La Foret en Jeu. L‘ Extractivisme en Amazonie Centrale. In: Emperaire L, editor. L' Orston: Institut Français de Recherche Scientifique pour le Développement en Coopéracion. Paris: Unesco; 1996.

3. Pindorama. São Paulo: Mercedes-Benz do Brazil; 1993.
4. Kahn F, Corradini L. Palmeiras da Amazonia. Ed. Cendotec. Brasilia: Centro Franco Brasileiro de Documentação Científica; 1994. Contato, special volume. p. 11-6.
5. Mapeamento e levantamento do potencial das ocorrências de babaçuais. Brasília: Núcleo de Comunicação Social da Secretaria de Tecnologia Industrial, Ministério da Indústria e do Comércio; 1982. p. 11-62.
6. Moomen H. Emerging infectious diseases—Brazil. Emerg Infect Dis 1998;4:351-4.
7. Brasil RP, Silva AR, Arbarelli A, Vale JF. Distribuição e infecção de triatomineos por *Trypanosoma* tipo *cruzi* na Ilha de São Luis, Maranhão. Rev Soc Bras Med Trop 1985;18:257-60.
8. Miles MA, Souza de AA, Póvoa M. Chagas diseases in the Amazon Basin. III. Ecotopes of ten triatomine bug species (Hemiptera: *Reduvidae*) from the vicinity of Belém, Pará State, Brazil. J Med Entomol 1981;18:266-78.
9. Miles MA, Arias JR, Souza AA. Chagas disease in the Amazon Basin. V. Peri-urban palms as habitats of *Rhodnius robustus* and *Rhodnius pictipes* triatominae vectors of Chagas disease. Mem Inst Oswaldo Cruz 1983;78:391-8.
10. Lainson R, Shaw JJ, Fraiha H, Miles MA, Draper CC. Chagas disease in the Amazon Basin: *Trypanosoma cruzi* infections in sylvatic mammals, triatomine bugs and man in the State of Pará, north Brazil. Trans R Soc Trop Med Hyg 1979;73:193-204.
11. Recommendations from a satellite meeting. In: International Symposium to commemorate the 90th anniversary of the discovery of Chagas disease. Rio de Janeiro: Mem Inst Oswaldo Cruz 1999;94:429-32.
12. D'Alessandro A, Barreto P, Saravia N, Barreto M. Epidemiology of *Trypanosoma cruzi* in the oriental plains of Colombia. Am J Trop Med Hyg 1984;33:1084-95.
13. Coura JR, Barret TV, Naranjo CM. Ataque de populações humanas por triatomineos silvestres no Amazonas: uma nova forma de transmissão da infecção Chagásica? Rev Soc Bras Med Trop 1994;27:251-3.
14. Valente VC, Valente SA, Noireau F, Carrasco HJ, Miles MA. Chagas disease in the Amazon Basin: association of *Panstrongylus geniculatus* (Hemiptera: Reduviidae) with domestic pigs. J Med Entomol 1998;35:99-103.
15. Amunárriz MU, Chico ME, Guderian RH. Chagas disease in Ecuador: a sylvatic focus in the Amazon Region. J Trop Med Hyg 1991;94:145-9.
16. Whitlaw JT, Chaniotis BN. Palm trees in Panama. Am J Trop Med Hyg 1978;27:873-81.
17. Almeida FB. Triatomíneos da Amazonia. Encontro de três espécies naturalmente infectadas por trypanosoma semelhante ao cruzi no Estado do Amazonas (Hemiptera, Reduviidae). Acta Amáz (Manaus) 1971;1:71-5.
18. Coura JR. Doença de Chagas como endemia na amazonia brasileira: risco ou hipótese? Rev Soc Bras Med Trop 1990; 23:67-70.
19. Fraiha Neto H, Valente SA, Valente VC, Pinto AYN. Doença de Chagas endemica na Amazonia. Rev Acad Med Pará, Belém 1995; 6:1-132.
20. Chagas C. Infection naturelle des singes du Pará (*Crysotrix sciureus*) par *Trypanosoma cruzi*. C R Soc Biol (Paris) 1924;9:873-6.
21. Raccurt CP. *Trypanosoma cruzi* in French Guinea: review of accumulated data since 1940. Med Trop 1996;56:79-87.
22. Shaw JJ, Lainson R, Fraiha H. Considerações sobre a epidemiologia dos primeiros casos autóctones de doença de Chagas registrados em Belém, Pará, Brasil. Rev Bras Saúde Pública, São Paulo 1969;3:153-7.
23. Ferraroni JJ, Melo JAN, Camargo ME. Molestia de Chagas na Amazônia. Ocorrência de seis casos suspeitos, autóctones, sorologicamente positivos. Acta Amazônica 1977;3:438-9.
24. Silva AR, Mendes JR, Mendonca ML, Cutrin RN, Brazil RP. Primeiros casos agudos autoctones da doença de Chagas no Maranhão e inquérito soro-epidemiologico da população. Rev Soc Bras Med Trop 1985;18:269-70.
25. Rodrigues CI, Souza AA, Terceros R, Valente S. Doença de Chagas na Amazonia: I Registro de oito casos autóctones em Macapa. Rev Soc Bras Med Trop 1988;21:193-7.
26. Acosta HMS, Ferreira CS, Carvalho ME. Human infection with *Trypanosoma cruzi* in Nasca, Peru: a seroepidemiologic survey. Rev Inst Med Trop São Paulo 1997;39:107-12.
27. Chico MH, Sandoval C, Guevara AE, Calvopiña MH, Cooper PJ, Reed SG, et al. Chagas disease in Ecuador: evidence for disease transmission in an indigenous population in the Amazon Region. Mem Inst Oswaldo Cruz 1997;92:317-20.
28. Corredor-Arjona A, Moreno CA, Agudelo CA, Bueno M, López MC, Cáceres E, et al. Prevalence of *Trypanosoma cruzi* and *Leishmania chagasi* infection and risk factors in a Colombian indigenous population. Rev Inst Med Trop São Paulo 1999;41: 229-4.
29. Spellerberg IF, Hardes S. Biological conservation. Cambridge: Cambridge University Press, 1992.
30. Williams-Blangero S, VandeBerg J, Blangero J, Teixeira ARL. Genetic epidemiology of seropositivity for *Trypanosoma cruzi* infection in rural Goias, Brazil. Am J Trop Med Hyg 1997;57: 538-43.
31. Pedreira de Freitas JL, Siqueira AF, Ferreira OA. Investigações epidemiológicas sobre triatomíneos de hábitos domésticos e silvestres com auxílio da reação de precipitina. Rev Inst Med Trop São Paulo 1960:2:90-9.
32. Siqueira AF. Estudos sobre a reação da precipitina aplicada à identificação de sangue por triatomineos. Rev Inst Med Trop São Paulo 1960;2:41-53.
33. Teixeira ARL. The Stercorarian trypanosomes. In: Immune responses in parasitic infections: immunology, immunopathology and immunoprophylaxis. EJL Soulsby, editor. Boca Raton: CRC Press, 1987. pp. 125-45.
34. Teixeira ARL, Teixeira G, Macedo V, Prata A. Acquired cell-mediated immunodepression in acute Chagas disease. J Clin Invest 1978;62:1132-8.
35. Sturm NR, Degrave W, Morel CM, Simpson L. Sensitive detection and schizodeme classification of *Trypanosoma cruzi* by amplification of kinetoplast minicircle DNA sequences: use in the diagnosis of Chagas disease. Mol Biochem Parasitol 1984;33:205-14.

36. Avila HA, Sigman DS, Coohen LM, Millikan RC, Simpson L. Polymerase chain reaction amplification of *Trypanosoma cruzi* kinetoplast minicircle DNA isolated from whole blood lysates: diagnosis of chronic Chagas disease. Mol Biochem Parasitol 1991;48:211-22.
37. Moser DR, Kirchhoff LV, Donelson J. Detection of *Trypansoma cruzi* DNA amplification using the polymerase chain reaction. J Clin Microbiol 1989;27:1477-82.
38. Fernandes O, Sturm NR, Campbell D. The mini-exon gene: a genetic marker for zymodeme III of *Trypanosoma cruzi*. Mol Biochem Parasitol 1998;95:129-33.
39. Briones MR, Souto RP, Stolf BS, Zingales B. The evolution of two *Trypanosoma cruzi* subgroups inferred from rRNA genes can be correlated with the interchange of American mammalian faunas in the Cenozoic and has implications to pathogenicity and host specificity. Mol Biochem Parasitol 1999;104:219-32.
40. Souto RP, Zingales B. Sensitive detection and strain classification of *Trypanosoma cruzi* by amplification of a ribosomal RNA sequence. Mol Biochem Parasitol 1993;63:45-52.
41. Souto RP, Vargas N, Zingales B. *Trypanosoma rangeli*: discrimination from *Trypanosoma cruzi* based on a variable domain from the large subunit ribosomal RNA gene. Exp Parasitol 1999;91:306-14.
42. Jorg ME. La modificación del biotopo peri-habitacional en la profilaxis de la enfermedad de Chagas. Rev Soc Bras Med Trop 1989;22:91-5.
43. Vexenat AC, Santana JM, Teixeira ARL. Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa *Trypanosoma cruzi*, *Leishmania chagasi* and *Leishmania (viannia) braziliensis*. Rev Inst Med Trop São Paulo 1996;38:177-85.
44. Guhl F, Hudson L, Marinkelle CJ, Jaramillo CA, Bridge C. Clinical *Trypanosoma rangeli* infection as a complication of Chagas disease. Parasitology 1987;94:475-84.
45. Romaña CA, Pizarro JC, Rodas E, Guilbert E. Palm trees as ecological indicators of risk areas for Chagas disease. Trans R Soc Trop Med Hyg 1999;93:594-5.
46. Barreto MP, Siqueira AF, Correa FMA, Ferrioli F, Cavalheiro JR. Estudos sobre reservatórios e vetores silvestres do *Trypanosoma cruzi*. VII: investigacões sobre a infecção natural de gambás por *Tripanossomos* semelhantes ao "*T. cruzi.*" Rev Bras Biol 1964;24:289-300.
47. Barreto MP, Siqueira AF, Filho, Cavalheiro JR. Estudos sobre reservatórios e vetores silvestres do *Trypanosoma cruzi.* XI: Observações sobre um foco da tripanosomose Americana no Município de Ribeirão Preto, São Paulo. Rev Inst Med Trop São Paulo 1966;8:103-12.
48. Barreto MP, Alburquerque RDR, Funayama GK. Estudos sobre reservatórios e vetores silvestres do *Trypanosoma cruzi.* XXXVI: investigações sobre triatomineos de palmeiras no Municipio De Uberaba, MG, Brasil. Rev Soc Bras Biol 1969; 29:577-88.
49. Barreto MP, Ribeiro RD, Rocha GM. Estudos Sobre reservatórios e vetores silvestres do *Trypanosoma cruzi*. LXIX: Inquérito preliminar sobre triatomíneos silvestres na região do triângulo Mineiro MG, Brasil. Rev Bras Biol 1978; 38:633-7.
50. Hawksworth DL, Kirk PM, Sutton DN. Dictionary of the fungi. 8th edition. International Mycological Institute. Cambridge: University Press; 1995. p. 76, 272.
51. Fungos em plantas no Brasil. Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de Recursos Genéticos e Biotecnologia, Ministério da Agricultura e do Abastecimento. Brasília: Serviço de Produção de Informação; 1988. p. 171.
52. Edwards PJ, Wratten SD. Ecologia das interações entre insetos e plantas. São Paulo: Editora Pedagógica e Universitária Ltda; 1981. Vol. 27.
53. Kempf WW. Levantamento das formigas da mata amazônica, nos arredores de Belém do Pará, Brasil. Studia Entomológica. Rio de Janeiro: Vozes Ltda; 1970. p. 321-43.
54. Naiff MF, Naiff RD, Barret TV. Vetores selváticos de doença de Chagas na área urbana de Manaus (AM): atividade de vôo nas estações secas e chuvosas. Rev Soc Bras Med Trop 1998; 31:103-5.
55. Miles MA, Arias JR, Naiff RD, Souza AA, Povoa MM, Lima JAN, et al. Vertebrate hosts and vectors of *Trypanosoma rangeli* in the Amazon Basin of Brazil. Am J Trop Med Hyg 1983; 32:1251-9.
56. Helmut S. Birds in Brazil. A natural history. New Jersey: Princeton University Press;1993.
57. Deane L. Animal reservoirs of *Trypanosoma cruzi* in Brazil. Rev Bras Malariol Doenças Tropicais 1964;16:28-48.
58. Deane MP, Jansen AM. Another *Trypanosoma,* distinct from *T. cruzi*, multiples in the lumen of the anal glands of the opossum *Didelphis marsupialis*. Mem Inst Oswaldo Cruz 1986; 81:131-2.
59. Jansen AM, Madeira F, Carreira JC, Acosta-Medina, Deane MP. *Trypanosoma cruzi* in the opossum *Didelphis marsupialis*: a study of the correlations and kinetics of the systemic and scent gland infections in naturally and experimentally infected animals. Exp Parasitol 1997;86:37-44.
60. Odun EP. Ecologia. Biblioteca Pioneira de Biologia Moderna. São Paulo: Livraria Pioneira; 1977. p. 19-148.
61. Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis 1995;1:7-15.
62. Valente AS, Valente VC, Fraiha Neto A. Considerations on the epidemiology and transmission of Chagas disease in the Brazilian Amazon. Mem Inst Oswaldo Cruz 1999;94:395-8.
63. Silveira AC, Rezende DF. Epidemiologia e controle da transmissão vetorial da doença de Chagas no Brasil. Rev Soc Bras Med Trop 1994;27(Supl III):11-22.
64. Silveira AC, Vinhaes MC. Elimination of vector-borne transmission of Chagas disease. Mem Inst Oswaldo Cruz 1999; 94:405-11.
65. Camargo ME, Silva GR, Castilho EA, Silveira AC. Inquérito sorológico da prevalência de infecção chagasica no Brasil, 1985-1980. Rev Inst Med Trop São Paulo 1984;26:192-204.
66. Dias JCP. A doença de Chagas e seu controle na América Latina. Uma análise de possibilidades. Rio de Janeiro: Cad Saúde Pública 1993;9:201-9.
67. Moncayo A. Chagas disease: epidemiology and prospects for interruption of transmission in the Americas. Wld Hlth Statist Quart 1992;45:276-9.

68. Valente VC. Potential for domestication of *Panstrongylus geniculatus* in the municipality of Muaná, Marajó Island, State of Pará, Brazil. Mem Inst Oswaldo Cruz 1999;94:399-400.
69. Feitosa VR. Implantação de um sistema de vigilância epidemológica (VE) de doença de Chagas na Amazônia. Ver Soc Brás Méd Trop 1995;28:84-7.
70. Coura JR, Junqueira ACV, Boia MN, Fernandes O. Chagas disease: from bush to huts and houses. Is it the case of the Brazilian Amazon? Mem Inst Oswaldo Cruz 1999;94:379-84.
71. Bos R. The importance of peridomestic environmental management for the control of the vectors of Chagas disease. Rev Argent Microbiol 1988;20:58-62.
72. Kroeger A, Ordonez-Gonzalez J, Behrend M, Alvarez G. Bednet impregnation for Chagas disease control: a new perspective. Trop Med Int Health 1999;4:194-8.
73. Wood E, de Licastro SA, Casabe N, Picollo MI, Alzogaray R, Nicolas Zerba E. A new tactic for *Triatoma infestans* control: fabrics impregnated with beta-cypermethrin. Rev Panam Salud Publica 1999;6:1-7.

Persistence and Variability of *Stenotrophomonas maltophilia* in Cystic Fibrosis Patients, Madrid, 1991–1998

Sylvia Valdezate,*,† Ana Vindel,† Luis Maiz,*
Fernando Baquero,* Hector Escobar,* and Rafael Cantón*
*Hospital Ramón y Cajal, Madrid, Spain; †Centro Nacional de Microbiología, Instituto Carlos III, Madrid, Spain

During 1991 to 1998 at least one *Stenotrophomonas maltophilia* pulmonary infection was observed in 25 (24%) of 104 cystic fibrosis patients at the same unit of our hospital in Spain. Ribotyping and pulse-field gel electrophoresis (PFGE) characterization of 76 *S. maltophilia* isolates from these patients indicated an overall clonal incidence of 47.1%, reflecting new strains in 44% of patients with repeated positive cultures for *S. maltophilia*. Six patients with repeated episodes were persistently colonized ($\geq$6 months) with the same strain. *S. maltophilia* bacterial counts were higher (geometric mean, 2.9 x10^8 cfu/mL) in patients with repeated episodes than in those with a single episode (8.4 x10^4 cfu/mL, $p<0.01$). Single episodes of *S. maltophilia* occurred in patients <10 years of age (43% [6/14]), whereas chronic colonization occurred more frequently in older patients (>16 years of age).

Pulmonary infection due to chronic microbial colonization is the major cause of illness and death in cystic fibrosis (CF) patients. Mucoid *Pseudomonas aeruginosa,* which is involved in pulmonary damage, is the most frequently recovered pathogen. In contrast, little information is available about the role of other nonfermentative gram-negative rods. An increasing incidence of *Stenotrophomonas maltophilia* isolates has been reported in some CF centers during the last decade (1-4). Although an association between *S. maltophilia* colonization and lung damage has been observed (2,3), the role of the organism is still undetermined (5,6). In non-CF patients (e.g., immunocompromised or intensive-care unit patients), exposure to wide-spectrum antimicrobial drugs, long-term antimicrobial therapy, previous pulmonary infections, and chronic respiratory disease contribute to *S. maltophilia* acquisition and increase the risk for respiratory infection with this organism (7,8). All these risk factors are present in the CF population.

Address for correspondence: Rafael Cantón, Servicio de Microbiología, Hospital Ramón y Cajal, Carretera de Colmenar, Km 9,100, 28034-Madrid, Spain; fax: 34-91-3368809; e-mail: rcanton@hrc.insalud.es.

We analyzed *S. maltophilia* from respiratory isolates of 25 CF patients of the same CF unit during an 8-year period to determine a) the overall and yearly incidence of *S. maltophilia* infection or colonization and incidence as determined by molecular typing, ribotyping, and pulsed-field gel electrophoresis (PFGE); b) the age distribution of acquisition of *S. maltophilia* pulmonary infection or colonization in patients with single or repeated episodes; c) the persistence and variability of *S. maltophilia* isolates in patients who had more than one episode and the degree of genomic similarity identified among clones; and d) the epidemiologic link between similar isolates from different patients. We also investigated pulmonary function and other clinical aspects of *S. maltophilia*-infected or colonized patients.

Materials and Methods

From 1991 to 1998, 25 CF patients (12 female and 13 male) of 104 who were clinically and microbiologically followed at the Hospital Ramón y Cajal CF Unit had at least one positive respiratory culture for *S. maltophilia*. CF was diagnosed by a positive sweat chloride test

(>60 mEq/L) in association with typical pulmonary and gastrointestinal findings or a positive family history. The age range of patients was <1 to 32 years (median 14.5 years). Eleven patients were homozygotes and eight heterozygotes for ΔF508, the most common mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and one patient was negative for ΔF508. Mutation in this gene could not be determined in five patients. The mean number of sputum samples examined was 6.7 specimens per patient per year. All 25 patients were followed for at least 1 year during the study period (range 1 to 8 years, mean 5.8 years). Culture results were used to establish age at acquisition of *S. maltophilia*. When available, retrospective cultures obtained before 1991 were also taken into account.

S. maltophilia colonization in CF patients was considered persistent if positive cultures were obtained for >6 months, regardless of bacterial counts. Overall incidence was defined as the number of patients infected or colonized with *S. maltophilia*, independent of the number of positive cultures during the study period. The denominator was the total number of patients seen at the CF unit (104 patients). Yearly incidence was defined as the number of patients with new episodes of *S. maltophilia* infection or colonization, with the denominator the number of patients seen per year in the CF unit. The overall incidence and yearly incidence were recalculated when molecular typing data were available. These values were defined as overall clonal incidence and yearly clonal incidence, respectively, which represent the incidence of *S. maltophilia* episodes caused by different clonal strains.

Bacteriologic Study and *S. maltophilia* Isolates

Respiratory secretions, mostly expectorated sputum, were homogenized with N-acetylcysteine and processed by a modified quantitative technique (9). Columbia 5% blood, MacConkey, mannitol and salt, and a selective *Burkholderia cepacia* agar media were incubated in air for 24 hours at 37°C, followed by 24 hours at 25°C. In addition, bacitracin-chocolate agar was plated and incubated in 5% CO_2 for 48 hours and Sabouraud-chloramphenicol and Sabouraud-chloramphenicol-cyclohexamide agar media for 4 weeks at 30°C and 37°C. A culture for *S. maltophilia* was considered positive when any growth of this organism was observed, regardless of bacterial count. Biochemical identification of *S. maltophilia* isolates was performed both with the API 20NE gallery (BioMerieux, Marcy-l´Étoile, France) and the semiautomatic PASCO system (Difco, Detroit, MI). Bacterial counts and co-colonization with other respiratory pathogens were also considered in the analysis. The same microbiologic protocol was applied to all patients, regardless of clinical condition.

Ribotyping

DNA from all *S. maltophilia* isolates was prepared by treatment with hexadecyltrimethylammonium bromide (10). Ribotyping was performed as described (11). *Bam*H1, *Bsu*15I, *Eco*RI, and *Hin*dIII restriction endonucleases (Roche Diagnostic, Mannheim, Germany) were also tested in a representative number of isolates. The best-defined restriction pattern with a higher number of bands was observed with *Bam*H1 and *Hin*dIII. Digoxigenin-labeled phage λ*Hin*dIII-digested DNA (Roche) was used as a molecular size marker. DNA fragments were separated by electrophoresis in 0.7% agarose gels and were blotted onto nylon membranes. Membranes were hybridized with a digoxigenin-labeled rRNA probe with 16S+23S rRNA sequences of *Escherichia coli* (Roche) at 68ºC for 18 hours (12). Differences in numbers and the position of bands were considered.

Pulsed-Field Gel Electrophoresis

S. maltophilia DNA was prepared and contained in agarose plugs for digestion with 30 U of *Xba*I (Roche). Closely related isolates using *XbaI* were reanalyzed with 20 U of *Spe*I (Roche) as described (13). Digested samples were melted and loaded onto 1% agarose gels. PFGE was performed with the CHEF-DRII system (Bio-Rad, Hemel Hempstead, UK). Standard lambda ladders comprising 48.5-kbp concatemers were run as molecular weight markers (Roche). Electrophoresis pulse times for *Xba*I-digested DNA were 10 to 60 seconds for 24 hours, followed by a second ramp from 5 to 20 seconds for 5 hours. Both ramps were performed at 5.4 V/cm and 12º C. For *Spe*I, pulse times were 25 to 45 seconds for 20 hours at 6 V/cm and 12°C. Macrorestriction fragments were visually compared and interpreted according to the criteria of Tenover et al. (14).

A genetic similarity dendogram was designed and calculated by the Dice correlation coefficient (15) and represented by UPGMA with Molecular

Analyst Software (BioRad) and a tolerance position of 1%. Only well-resolved bands corresponding to fragments exceeding 97.0 kbp were included in the computer analysis.

Patient Data

Chart records from *S. maltophilia*-positive CF patients were reviewed. Patients were classified according to age, sex, and severity of lung disease. Correlation between colonization or infection with *S. maltophilia* and pulmonary function was studied. Pulmonary function was tested in accordance with American Thoracic Society Guidelines (16). Forced expiratory volume (FEV_1) (% predicted) value was expressed as the percentage predicted according to Knudson norms for adjusting data for age, height, and sex (17). Trends in FEV_1 were estimated by comparing values at the time of the first recovery of *S. maltophilia* with those obtained within a year from the last isolation. *P. aeruginosa* and other pathogens commonly encountered in CF were also recorded as outcome criteria for evaluating the progression of pulmonary disease.

Statistic Analysis

Statistical significance for comparison proportions was calculated by Chi square or Fisher's exact test with Epi-Info 6.04a. Quantitative values were compared by Student's *t* test; $p<0.05$ was considered statistically significant.

Results

From 1991 to 1998, at least one respiratory culture positive for *S. maltophilia* was observed in 25 of 104 patients. Thus, the overall incidence of *S. maltophilia*-infected or -colonized patients was 24%; yearly incidence was 2.9% to 14.0% (Figure 1). Fourteen (56%) of these 25 patients had a single episode of *S. maltophilia* (SM-SE group), and 11 patients (44%) had repeated episodes (SM-RE group). No differences in sampling frequency (number of sputum samples studied per year) or length of follow-up were found between the two groups.

Eighty-eight *S. maltophilia* isolates were recovered from these 25 patients. Seventy-six isolates, 14 from the SM-SE group and 62 from the SM-RE group, were available for further study. PFGE results indicated an overall clonal incidence of 47.1%, reflecting new strains with different PFGE profiles that had been acquired by the SM-RE group (Figure 1). The highest yearly clonal incidences were detected in 1991 and 1996.

In the SM-SE group, the median age at acquisition of *S. maltophilia* was 13.4 years (range <1 to 27 years). Nearly 43% of patients (6 of 14) acquired *S. maltophilia* at 6 to 10 years of age (Figure 2). In SM-RE patients, the median age at first *S. maltophilia* isolation was 16.7 years (range 3 to 32 years). In this group, 45% (5 of 11) acquired *S. maltophilia* at 11 to 20 years of age. PFGE analysis of all *S. maltophilia* strains

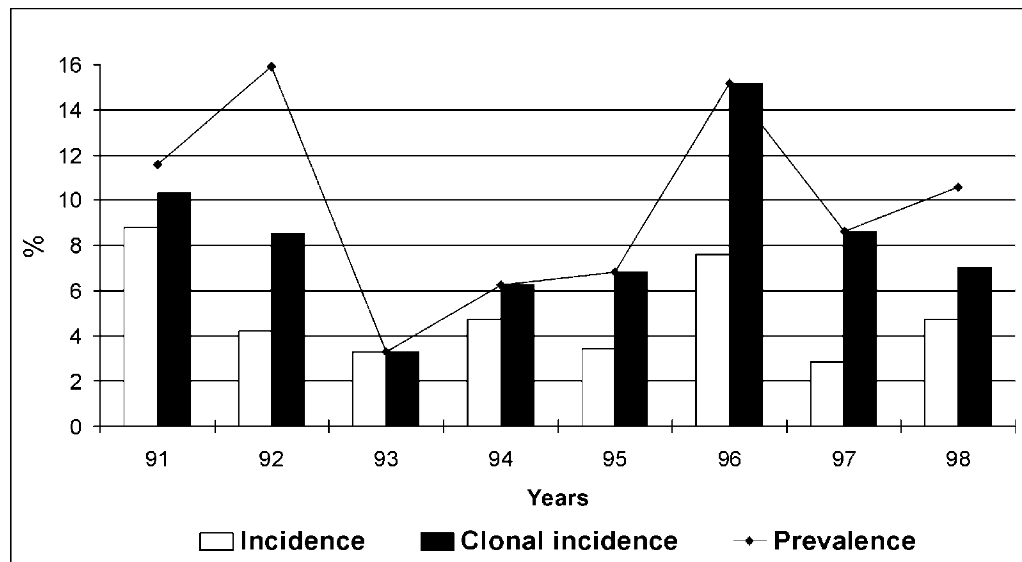


Figure 1. Yearly incidence, yearly clonal incidence, and yearly prevalence of *Stenotrophomonas maltophilia* acquisition in 104 cystic fibrosis patients, 1991–1998.

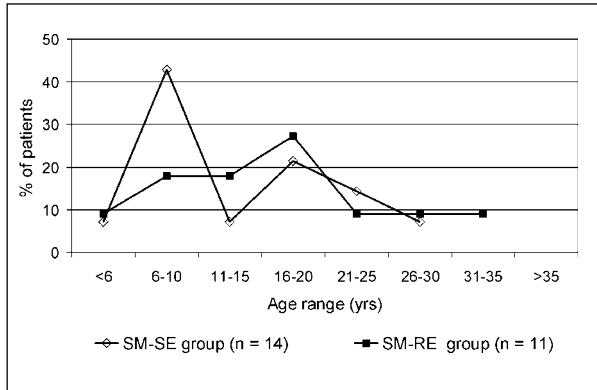


Figure 2. Distribution of age of acquisition of *Stenotrophomonas maltophilia* in 25 cystic fibrosis patients with a single episode (SM-SE group) or with repeated episodes (SM-RE group).

indicated that nine new acquisitions occurred in 11- to 15-year-old patients. Because of the small sample size, differences in age of acquisition between the groups could not be demonstrated with statistical significance.

Ribotyping

To select the suitable enzyme(s) for *S. maltophilia* ribotyping, *Bam*H1, *Bsu*15I, *Eco*RI, and *Hin*dIII endonucleases were used in five different strains isolated from the same patient, resulting in 4, 4, 2, and 4 different ribotypes, respectively. The number of copies of the ribosomal rRNA operon in *S. maltophilia* was 2 to 5 per isolate for *Bam*HI, 2 to 4 *Bsu*15I for *Hin*dIII, and 4 to 5 for *Eco*RI, with hybridization band sizes of 3 Kbp to 20 kbp. Great heterogeneity in ribotypes, 21 with *Hin*dIII and 20 with *Bam*HI, was found among the 76 *S. maltophilia* isolates, with a Simpson index (15) of 0.8992 and 0.9158, respectively. The genetic similarity was 29% to 100% for *Hin*dIII and 38% to 100% for *Bam*HI.

PFGE Analysis

Forty-seven well-defined profiles of genomic DNA under *Xba*I digestion were obtained from the 76 *S. maltophilia* isolates. According to Tenover criteria (14), 41 types and 6 subtypes were considered. These 6 subtypes were associated with 3 of the 41 main subtypes. Fragment size was <48 kbp to >1,000 kbp. Discrimination based on Simpson´s index peaked at 0.97. Genetic heterogeneity is illustrated by the dendogram of the 47 *Xba*I-PFGE profiles (Figure 3). Repeated isolates displaying an identical PFGE profile from

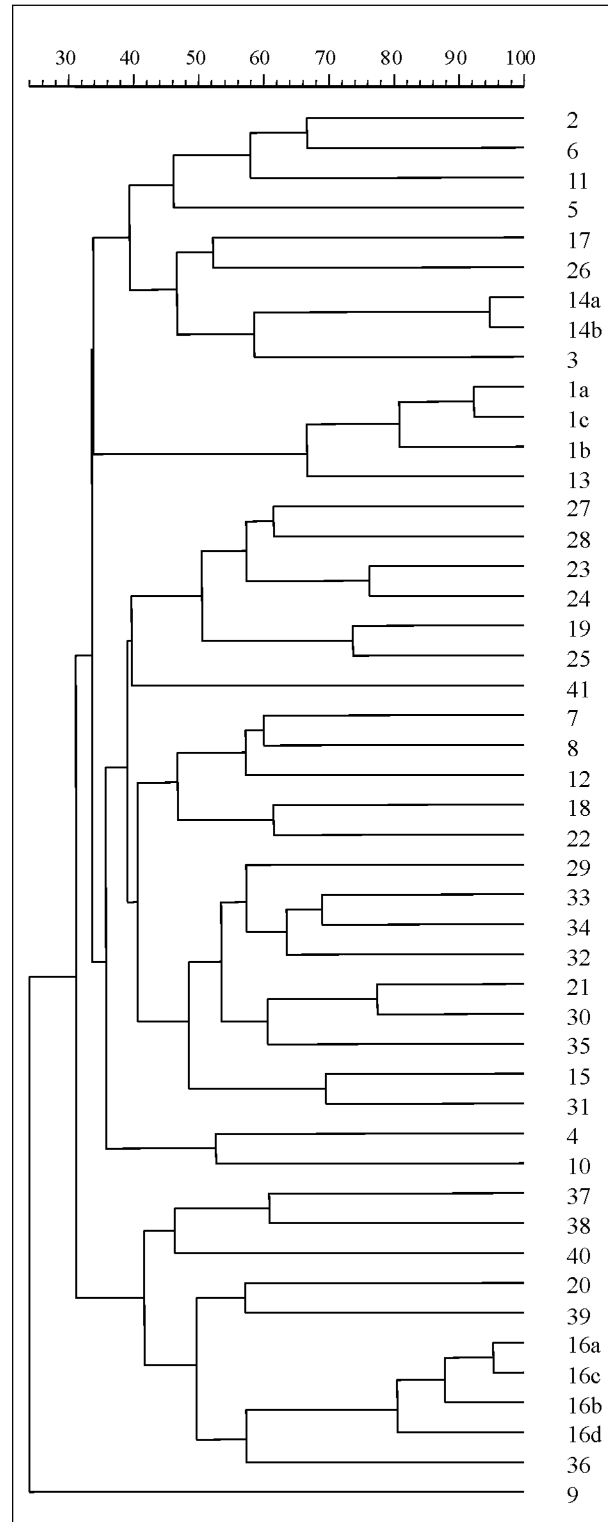


Figure 3. Percentages of genetic similarity between 47 *Xba*I-PFGE clonal types from 76 *Stenotrophomonas maltophilia* strains isolated in 25 cystic fibrosis patients of the same unit, 1991–1998.

the same patient or resulting from presumed patient-to-patient transmission were excluded from the dendogram. Forty-one types displayed similarity coefficients from 25% to 75%; each was coded with a number. Each subtype was coded with a letter (similarity ≥80%). Strains sharing the same *Xba*I digestion pattern could not be further distinguished by *Spe*I. *Xba*I was more efficient than *Spe*I in distinguishing between subtypes or closely related strains; 14a and 14b subtypes showed an indistinguishable PFGE pattern with *Spe*I. This was also the case with 16a and 16c subtypes.

Persistence and Variability of *S. maltophilia* Strains

The SM-SE group of 14 patients had 14 different PFGE types. One of these PFGE patterns (pattern *1a*) was also seen in two of the SM-RE patients (patients 1 and 3). During the study period, each of the 11 patients in the SM-RE group had one to five strains with different PFGE profiles. Strains from five patients (1, 3, 8, 10, and 11) completely met the criteria for persistence (Figure 4). The strains were recovered from these patients during periods of persistence of 29, 86, 6, 9, and 8 months, respectively. A turnover of this predominant strain with a different strain occurred in four of these patients. In patient 4, two strains with 4 and 32 months of persistence were isolated during two different periods. All these patients were considered persistently colonized with identical *S. maltophilia* isolates (Figure 4). Variable colonization, defined as the isolation of *S. maltophilia* strains with different PFGE

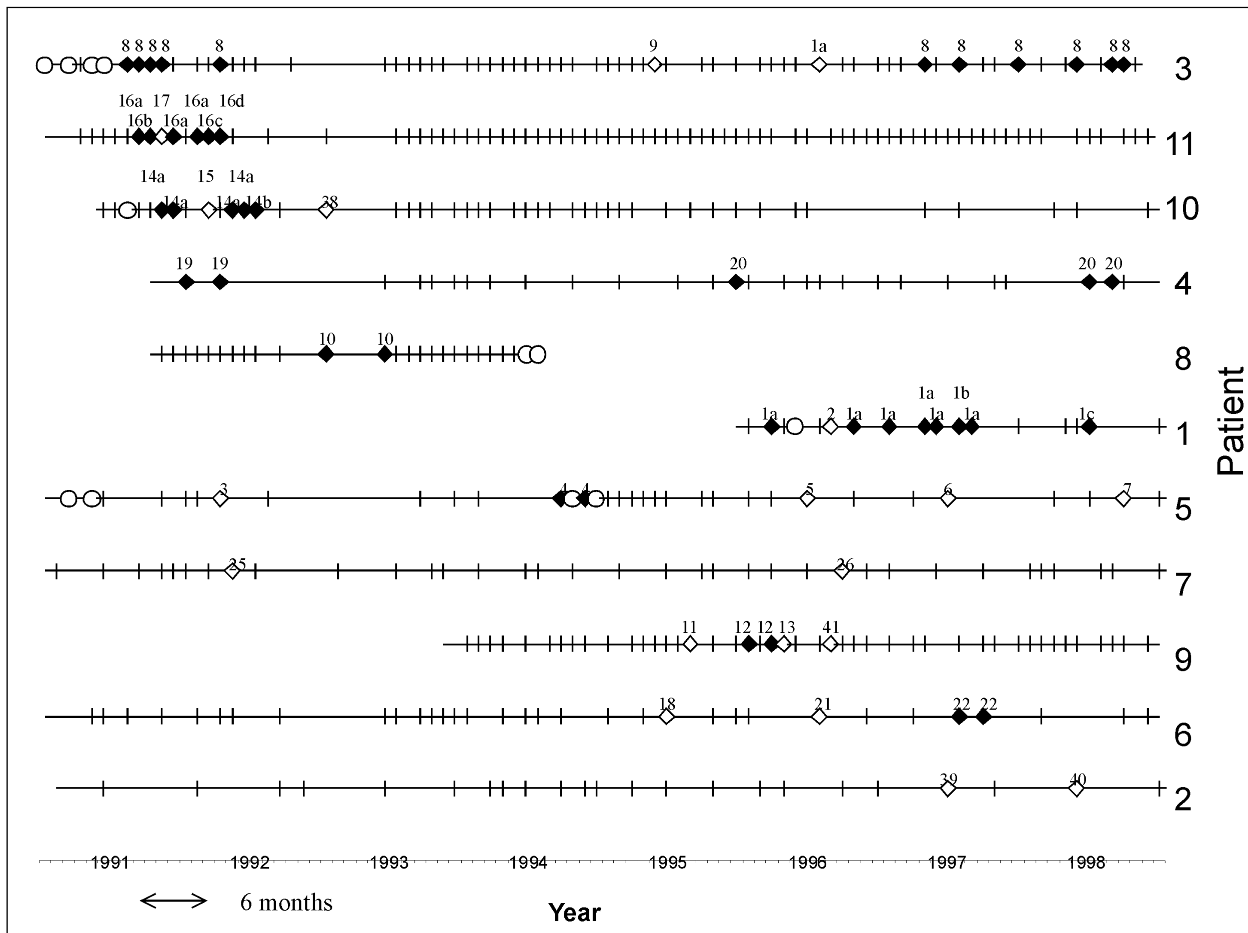


Figure 4. Persistence and variability of *Stenotrophomonas maltophilia* strains in 11 patients with repeated episodes of infection or colonization with this organism. *S. maltophilia* recovered is indicated by a ♦ (identical isolates from the same patient), ◊ (different isolate from a single patient) or O (no isolate available). Distinct *S. maltophilia* isolates are identified by numbers, and a letter was added when a closely related strain was observed. + = time at which sputum specimens were obtained.

profiles, was identified in five patients (patients 2, 5, 6, 7, and 9).

Suspected Cross-Transmission

In 1996, three patients, two in the SM-RE group (patients 1 and 3) and another (patient 12) in the SM-SE group, shared *S. maltophilia* isolates with indistinguishable ribotype and PFGE type under all restriction enzymes tested (profile *1a*). Patient 1 was persistently colonized with this strain for 2 years, and patient 3 was transiently colonized (Figure 4).

Bacterial Counts and Clinical Findings

SM-SE patients had higher *S. maltophilia* bacterial counts when either the first *S. maltophilia* isolate (geometric mean, 4.3 x10^5 cfu/mL) or all isolates (2.9 x10^8 cfu/mL) were taken into account ($p<0.05$), compared with patients with a single episode (8.4 x10^4 cfu/mL). A similar rate of *P. aeruginosa* recovered from the respiratory tract during the study period was noted in both groups (Table). In contrast, *Aspergillus* spp. was detected more frequently in the SM-RE group of patients. No statistical differences were found when co-colonization was evaluated. However, *S. maltophilia* co-colonization with *Aspergillus* spp. in the SM-RE group had a risk ratio of 3.8 compared with the SM-SE group.

Demographic and selected medical characteristics and results of respiratory tract cultures were analyzed for *S. maltophilia*-infected or -colonized patients (Table). Before *S. maltophilia* colonization, slightly lower pulmonary function levels (FEV_1, % predicted) were observed in patients with a single *S. maltophilia* episode than in patients with repeated episodes (Table).

Table. Demographic characteristics and co-colonization status of cystic fibrosis patients with *Stenotrophomonas maltophilia* infection or colonization

Characteristics	SM-SE[a] (14 patients)	SM-RE[b] (11 patients)
Gender		
Male	8	5
Female	6	6
Patient genotyped	11	9
Homozygous ΔF508	8	3
Heterozygous ΔF508	3	5
Other	0	1
Mean age at first *S. maltophilia* isolation (SD, years)	13.4 (7.3)	16.7 (7.4)
FEV_1[c] measured	11	10
FEV_1 (% predicted) before *S. maltophilia* recovery (Mean [SD])	68.7 (29.6)	74.2 (28.3)
≥100	2	1
70–99	3	5
40–69	4	3
<40	2	1
FEV_1 (% predicted) after *S. maltophilia* recovery (Mean [SD])	63.8 (20.7)	62.9 (24.2)
ABPA[c] condition	2	1
Death (%)	4 (28.5)	2 (18.2)
S. maltophilia bacterial counts (geometric mean, cfu/mL)	8.4 x 10^4 [d]	2.9 x 10^8 [d]
Pseudomonas aeruginosa detected (%)	12 (85.7)	9 (81.8)
Aspergillus detected (%)	7 (50.0)	7 (63.6)
S. maltophilia co-colonization with:[e]		
Only *S. maltophilia* detected (%)	1 (7.1)	1 (9.1)
P. aeruginosa (%)	8 (57.1)	3 (27.2)
Staphylococcus aureus (%)	6 (42.8)	3 (27.2)
Burkholderia cepacia (%)	1 (7.1)	1 (9.1)
Aspergillus spp. (%)	1 (7.1)	3 (27.2)
Candida (%)	3 (21.4)	2 (18.2)

[a]SM-SE = CF patients with a single episode of *S. maltophilia* colonization.
[b]SM-RE: CF patients with repeated episodes of *S. maltophilia* colonization.
[c]FEV = Forced expiratory volume.
[d]ABPA: allergic bronchopulmonary aspergillosis.
[e]$p<0.05$ comparing both groups.
[f]Patients in the SM-RE group colonized with organisms in addition to *S. maltophilia*. When different co-colonizations occurred in the same patient, we recorded only the cocolonization that was at least twice as frequent.

This value decreased in SM-RE patients from 74.2 ± 28.3 (mean value ± SD) (first isolation of *S. maltophilia*) to 62.9 ± 24.2 (last isolation of *S. maltophilia*), which could indicate a decreasing trend in FEV_1 after the first episode. However, this difference was not statistically significant. On the other hand, SM-SE patients had a higher death rate (28.5%) than the SM-RE group, but death rates in both groups were higher than those observed in *S. maltophilia*-negative patients (12.6%).

Conclusions

S. maltophilia, an essentially environmental organism, is the fourth organism in prevalence in bronchial secretions of CF patients, after *P. aeruginosa, Staphylococcus aureus,* and *Haemophilus influenzae* (5,18). Since it was first reported in CF patients in 1979 (19), this organism has been investigated for its role in the progression of CF pulmonary disease (5), and consensus documents have emphasized the importance of clinical microbiology laboratories in detecting its presence in CF respiratory secretions (20). Despite some virulence factors shared with *P. aeruginosa*, its potential for pathogenicity remains uncertain (21). We have reported a high incidence of *S. maltophilia*-colonized CF patients (30.7%) over a 5-year period (3), but, as in other studies (2,6,22), we did not address (through epidemiologic typing studies) whether this high rate was a consequence of patient-to-patient transmission or whether bacterial colonization was sporadically or chronically established.

The 1997 Cystic Fibrosis Foundation Patient Registry from the United States (18), which included 17,996 CF patients in a cross-sectional study analyzing one respiratory sample per patient per year, showed a percentage of positive cultures for *S. maltophilia* of 5.1%, a value slightly higher than in 1996 (3.9%) and 1995 (3.4%). In our study, the overall incidence, 24%, is higher than that observed in other studies (10.6% to 16.6%) with a similar length of follow-up (2,22), but slightly lower than in studies with a longer follow-up period (27.3%) (6). Consistent with other results, our data showed no clear trend towards increasing or decreasing over the study period (Figure 1).

The main purpose of our study was to apply molecular typing, both with ribotyping and PFGE, to *S. maltophilia* isolates recovered from patients seen in our CF Unit. Among 76 isolates, 47 PFGE profiles were identified, and these results were used to calculate the incidence of episodes of *S. maltophilia* colonization or infection in our series. Without typing, the overall incidence was 24% for the entire study period; by PFGE the incidence was 47.1%. This result clearly indicates that SM-RE patients had new episodes with different *S. maltophilia* strains. Molecular typing also differentiated patients who were chronically infected or colonized with the same strain (persistence) from those with repeated episodes with different *S. maltophilia* strains (variability).

PFGE has been recommended for epidemiologic studies of *S. maltophilia* isolates (13,23-25). The technique has been shown to be more discriminatory than enterobacterial repetitive intergenic consensus polymerase chain reaction and other molecular techniques for differentiation within this species (13). In our study, restriction endonuclease *Xba*I provides discriminatory patterns, with a high discrimination value on Simpson´s index (0.97), enabling easy interpretation of banding profiles. This enzyme has been used to study the stability of *S. maltophilia* from a CF patient over a 15-month period (26), the relationship between CF and environmental *S. maltophilia* isolates (13), and the epidemiology of *S. maltophilia* isolates from a hematology department (27). Other studies have been based on *Dra*I (25,28,29) and *Spe*I (23,27,30). In our study, *Xba*I was more efficient than *Spe*I in distinguishing between subtypes or closely related strains.

We observed only one positive culture of *S. maltophilia* over the study period in 14 patients, in accordance with the results of Demko et al. (6), who showed that 50% of CF patients had only one positive culture of *S. maltophilia* over a 13-year period. In contrast, 11 patients (44%) from our CF unit had repeated episodes of *S. maltophilia* colonization or infection. Typing studies, however, demonstrated different strains in five patients and, with the exception of patient 8, a persistent strain was characterized in the remaining six patients, but with a turnover with distinct strains (Figure 4). Because of sampling bias, some of these patients may also have had persistent colonization. Of 11 patients with repeated SM-RE isolates, 6 had evidence of persistent colonization (Figure 5). More frequent sampling could have increased this proportion.

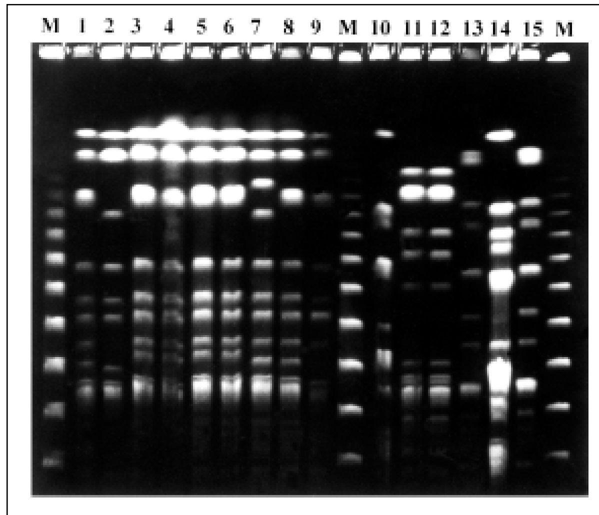


Figure 5. Pulsed-field gel electrophoresis pattern of *Xba*I-digested genomic DNA of *Stenotrophomonas maltophilia* isolates from two SM-RE patients. Lanes 1-10 from patient 1 (persistence group): pattern 1a (lanes 1,3-6,8), pattern 1b (ln 7), pattern 1c (lane 9) and pattern 2 (lane 2); lanes 10-15 from patient 5 (variability group): pattern 3 (lane 10), pattern 4 (lane 11,12), pattern 5 (lane 13), pattern 6 (lane 14), pattern 7 (lane 15). Lanes M, bacteriophage lambda standard marker.

Cross-transmission was suspected in three patients who shared isolates with an identical PFGE profile. No overlapping hospitalizations, clinical visits, or other epidemiologic relationship were demonstrated in these patients. Recently, Alfieri et al. (30) reported cross-transmission of *S. maltophilia* in non-CF patients during two consecutive nosocomial outbreaks in an intensive care unit, but an environmental ventilator isolate was temporally associated with infection.

Heterogeneity is also illustrated among *S. maltophilia* isolates recovered from the same patient. SM-RE patients 2, 5, 6, 7, and 9 were colonized at different times by different clones with PFGE similarity genetic coefficients of 24% to 61%. Among *S. maltophilia* isolates recovered from different patients, the genetic coefficient range was even wider (25% to 75%). This heterogeneity could result from acquisition from different environmental sources, probably outside the nosocomial setting. In fact, a high diversity of *S. maltophilia* isolates has also been confirmed in the environment (13,25). The precise mode of acquisition of *S. maltophilia* in CF patients has not been determined, but different studies strongly suggest that faucets, ventilators, sink drains, and other devices frequently in contact with water could be common sites of contamination (13,25,28,30,31).

In most cases, chronic colonization with *P. aeruginosa* occurs with a single strain, which undergoes phenotypic variation over time (32). This changing adaptive response is probably driven by stressful conditions of the lung environment for bacterial organisms and results from the selection of hypermutable genetic variants (33). In the case of *S. maltophilia*, the isolation of the same clonal type after years of apparent absence suggests a long low-grade persistence that could not be detected by microbiologic culture. In patient 3, the same strain was isolated 11 times over a 7-year period without change in its PFGE profile. The differing subtypes in patients 1, 10, and 11 may be accounted for by genetic events during chronic colonization (Figure 4).

The 1997 Cystic Fibrosis Patient Registry Annual Report (18) showed that *S. maltophilia* respiratory colonization was 3.1% to 8.6% in patients 2 to 5 and >45 years of age, respectively, with a clear increase in patients >35 years of age. We analyzed the age at first acquisition of an *S. maltophilia* isolate, including all 25 patients with at least one positive culture for this organism during the study period. When available, a retrospective review of cultures obtained before 1991 was also taken into account. Colonization rates were 4% to 24% in the 31-35 and 16-20 age groups, respectively. The peak age of acquisition was 16-20 years, as reported by Demko et al. (6), but the two groups of *S. maltophilia*-colonized patients, SM-SE and SM–RE, differed in age of acquisition. In SM-SE patients, peak age of acquisition was 6 to 10 years (42.8%); in the SM–RE group it was 16 to 20 years (27.2%). These results suggest that *S. maltophilia* colonization in younger CF patients could be an isolated event, whereas chronic colonization with this organism occurs more frequently when acquired in 16- to 20-year-old patients.

Higher significant ($p<0.05$) differences in *S. maltophilia* bacterial counts were obtained in patients persistently colonized with this organism compared with those with single episodes, suggesting that the colonizing ability of a given strain may be a marker for future persistence. In addition, the former group had a decline in pulmonary function as indicated by FEV_1 (% predicted) values closest to the first and last *S. maltophilia* isolations. Reduction in pulmonary

function could also reflect increased age or the effect of other pathogens. In fact, a higher rate of *Aspergillus* spp. isolation was detected in CF patients chronically colonized with *S. maltophilia*. However, a higher death rate was observed in patients with a single episode of *S. maltophilia* (28.5%) than in patients with repeated episodes (18.2%), but both these values were higher than those obtained in *S. maltophilia*-negative patients (12.6%). Demko et al. recently reported a lower death rate in patients with long-term chronically *S. maltophilia*-positive cultures (7.7%) than in those with transient or acute positive cultures (21.1%)(6). Moreover, the combined death rate in *S. maltophilia*-positive patients (19.0%) was slightly higher than in *S. maltophilia*-negative patients (16.5%). In contrast, Goss et al. (34) demonstrated in a cohort study that *S. maltophilia* acquisition did not decrease survival in patients with CF, but patients with this organism had significantly lower FEV_1 (% predicted) values. These data suggest that isolation and persistence of *S. maltophilia* could contribute to a progression of clinical deterioration, particularly in patients with lower pulmonary function. Increased *S. maltophilia* colonization may be observed in the future as a result of improvements in life expectancy.

This work was supported by research grant 2114/98 from the Consejería de Educación y Cultura of the Comunidad de Madrid, Spain.

Dr. Valdezate is a fellow in the Clinical Microbiology Department at the Ramón y Cajal Hospital and at the Centro Nacional de Microbiología (Instituto Carlos III) in Madrid, Spain. Her research interests focus on epidemiology and resistance of *Stenotrophomonas maltophilia*, mainly from cystic fibrosis patients.

References

1. Gladman G, Connor PJ, Williams RF, David TJ. Controlled study of *Pseudomonas maltophilia* in cystic fibrosis. Arch Dis Child 1993;67:192-5.
2. Karpati F, Malmborg AS, Alfredsson H, Hjelte L, Strandvik B. Bacterial colonization with *Xanthomonas maltophilia*: a retrospective study in a cystic fibrosis patient population. Infection 1994;22:258-63.
3. Ballestero S, Virseda I, Escobar H, Lucrecia L, Baquero F. *Stenotrophomonas maltophilia* in cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 1995;14:728-79.
4. Burns JL, Emerson J, Stapp JR, Yim DL, Krzewinski J, Louden L, et al. Microbiology of sputum from patients at cystic fibrosis centers in the United States. Clin Infect Dis 1998;27:158-63.
5. Denton M. *Stenotrophomonas maltophila*: an emerging problem in cystic fibrosis patients. Rev Med Microbiol 1997;8:15-9.
6. Demko CA, Stern RC, Doershuk CF. *Stenotrophomonas maltophilia* in cystic fibrosis: incidence and prevalence. Pediatr Pulmonol 1998;25:304-8.
7. Villarino ME, Stevens LE, Schable B, Mayers G, Miller JM, Burke JP, et al. Risk factors for epidemic *Xantomonas maltophilia* infection/colonization in intensive care unit patients. Infect Control Hosp Epidemiol 1992;13:201-6.
8. Van Couwenberghe CJ, Farver TB, Cohen SH. Risk factors associated with isolation of *Stenotrophomonas (Xantomonas) maltophilia* in clinical specimens. Infect Control Hosp Epidemiol 1997;18:316-21.
9. Wong K, Roberts MC, Owens L, Fife M, Smith AL. Selective media for the quantification of bacteria in cystic fibrosis sputum. J Med Microbiol 1984;17:113-9.
10. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. New York: Greene Publishing Associates and Wiley Interscience; 1991(Suppl. 13):241-5.
11. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; 98:503-17.
12. Stull TL, LiPuma JJ, Edlind TD. A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA. J Infect Dis 1988;157:280-6.
13. Denton M, Todd NJ, Kerr KG, Hawkey PM, Littlewood JM. Molecular epidemiology of *Stenotrophomonas maltophilia* isolated from clinical specimens from patients with cystic fibrosis and associated environmental samples. J Clin Microbiol 1998;36:1953-8.
14. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulse-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233-9.
15. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988;26:2465-6.
16. American Thoracic Society. Standardization of spirometry: 1987 update. Am Rev Respir Dis 1987;136:1285-98.
17. Knudson RJ, Lebowitz MD, Holdberg CJ, Burrows B. Changes in the normal maximal expiratory flow-volume curve with growth and aging. Am Rev Respir Dis 1983;127:725-34.
18. National CF Patient Registry 1997 Annual Report. Bethesda, MD: Cystic Fibrosis Foundation; 1998.
19. Blessing J, Walker J, Maybury B, Yeager AS, Lewiston N. *Pseudomonas cepacia* and *Pseudomonas maltophilia* in cystic fibrosis patients [abstract]. Am Rev Respir Dis 1979;119:262.
20. Gilligan P. Report on the consensus document for microbiology and infectious diseases in cystic fibrosis. Clin Microbiol Newsletter 1996;18:83-7.
21. Denton M, Kerr KG. Microbiology and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clin Microbiol Rev 1998;11:57-80.
22. Gladman G, Connor PJ, Williams RF, David TJ. Controlled study of *Pseudomonas cepacia* and *Pseudomonas maltophilia* in cystic fibrosis. Arch Dis Child 1992;67:192-5.

23. Laing FPY, Ramotar K, Read RR, Alfieri N, Kureishi A, Henderson EA, et al. Molecular epidemiology of *Xanthomonas maltophilia* colonization and infection in the hospital environment. J Clin Microbiol 1995;33:513-8.
24. Marty N. Epidemiological typing of *Stenotrophomonas maltophilia*. J Hosp Infect 1997;36:261-6.
25. Berg G, Roskot N, Smalla K. Genotypic and phenotypic relationships between clinical and environmental isolates of *Stenotrophomonas maltophilia*. J Clin Microbiol 1999;37:3594-600.
26. Wüst J, Frei R, Gunthard H, Altwegg M. Analysis of restriction fragment length polymorphism and ribotyping of multirresistant *Stenotrophomonas maltophilia* isolated from persisting lung infection in a cystic fibrosis patient. Scand J Infect Dis 1995;27:499-502.
27. Fabe C, Rodríguez P, Cony-Makhoul P, Parneix P, Bebear C, Maugein J. Typage moleculaire par electrophorese en champ pulse de souches de *Stenotrophomonas maltophilia* isolees dans un service d'hematologie. Pathol Biol Paris 1996; 44:435-41.
28. Talon D, Bailly P, Leprat R, Godard C, Deconnink E, Cahn J-Y, et al. Typing of hospital strains of *Xanthomonas maltophilia* by pulse-field gel electrophoresis. J Hosp Infect 1994;27:209-17.
29. Yao JD, Conly JM, Krajden M. Molecular typing of *Stenotrophomonas (Xanthomonas) maltophilia* by DNA macrorestriction analysis and random amplified polymorphic DNA analysis. J Clin Microbiol 1995;33:2195-8.
30. Alfieri N, Ramotar K, Armstrong P, Spornitz ME, Ross G, Winnick J, et al. Two consecutive outbreaks of *Stenotrophomonas maltophilia (Xanthomonas maltophilia)* in an intensive-care unit defined by restriction fragment-length polymorphism typing. Infect Control Hosp Epidemiol 1999;20:553-6.
31. Weber DJ, Rutala WA, Blanchet CN, Jordan M, Gergen MF. Faucet aerators: A source of patient colonization with *Stenotrophomonas maltophilia*. Am J Infect Control 1999;27: 59-63.
32. Govan JRW, Nelson JW. Microbiology of lung infection in cystic fibrosis. Br Med Bull 1992;48:912-30.
33. Oliver A, Cantón R, Campo P. Baquero F, Blázquez J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis. Science 2000;288:1251-3.
34. Goss CH, Aitken ML, Johnson WC, Campbell PW, Rubenfeld GD. Acquiring *Stenotrophomonas maltophilia* does not decrease survival in patients with cystic fibrosis. Pediatr Pulmunol 1999(Suppl 19):334-335 (abstract)

Hospital Control and Multidrug-Resistant Pulmonary Tuberculosis in Female Patients, Lima, Peru

Field F. Willingham*†‡ Tracy L. Schmitz,‡§ Macbeth Contreras,‡ Sheela E. Kalangi,‡¶ Aldo M. Vivar,‡ Luz Caviedes,# Eduardo Schiantarelli, Paola Maurtua Neumann,†‡ Caryn Bern,‡‡ Robert H. Gilman,†‡# and the Working Group on TB in Peru[1]**
*University of Maryland, School of Medicine, Baltimore, Maryland, USA; †Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland, USA; ‡Proyectos en Informática, Salud, Medicina, y Agricultura (PRISMA), Lima, Peru; §Tufts University School of Medicine, Boston, Massachusetts, USA; ¶University of Arizona School of Public Health, Phoenix, Arizona, USA; #Universidad Peruana Cayetano Heredia, Lima, Peru; **Arzobispo Loayza Hospital, Lima, Peru; ‡‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

We examined the prevalence of tuberculosis (TB), rate of multidrug-resistant (MDR) TB, and characteristics of TB on a female general medicine ward in Peru. Of 250 patients, 40 (16%) were positive by sputum culture and 27 (11%) by smear, and 8 (3%) had MDRTB. Thirteen (33%) of 40 culture-positive patients had not been suspected of having TB on admission. Six (46%) of 13 patients whose TB was unsuspected on admission had MDRTB, compared with 2 (7%) of 27 suspected cases (p=0.009). Five (63%) of 8 MDRTB patients were smear positive and therefore highly infective. In developing countries, hospital control, a simple method of reducing the spread of MDRTB, is neglected.

From 1990 to 2000, tuberculosis (TB) caused an estimated 88 million new infections and 30 million deaths worldwide (1). In Peru, tuberculosis is highly endemic; a shantytown in Lima had an annual incidence of pulmonary tuberculosis of 364 per 100,000 population (2). Despite the implementation of community-based treatment and control programs in Peru (3), management of the disease has been complicated by high rates of multidrug-resistant (MDR) TB. In one study in Peru, 4.5% of all reported cases were resistant to isoniazid and rifampin (4). Nosocomial spread of MDRTB has been reported in both industrialized and developing countries and has been linked to inadequate hospital infection control practices (5-7).

Address for correspondence: Robert H. Gilman, Department of International Health, Johns Hopkins University School of Hygiene and Public Health, 615 North Wolfe St., Baltimore, MD 21205, USA; fax: 410-550-6733; e-mail: rgilman@jhsph.edu.

We investigated the potential for nosocomial spread of MDRTB in one city hospital in Lima. We assessed the prevalence of TB among hospitalized patients on a general medicine ward, the rate of MDRTB and the extent to which active pulmonary TB had been suspected in patients at the time of admission.

Methods

Study Population and Design

The study was conducted from January to December 1997 in the Arzobispo Loayza Hospital, an urban public hospital in Lima, Peru. This hospital was founded as a women's hospital in the eighteenth century and continues to serve

[1]Other members of the Working Group on TB in Peru include Oswaldo Bisbal, Anna Bowen, Rosa Cama, William Checkley, Scott Franzblau, Luis Miguel Frachie, Patricia Fuentes, Hugo Garcia, Guillermo Lescano, Cheryl Liechty, Sonia Montenegro, Guillermo Salazar, Patricia Sheen, Eduardo Ticona, Teresa Valencia, and Richard Witzig.

a largely female patient population. We solicited the participation of all patients admitted to one of the hospital's eight female internal medicine wards (an open room with 30 beds) during the study period. The most common admission diagnoses over the year of study were pneumonia, bronchiectasis, cardiac insufficiency, TB, cellulitis, diabetes mellitus and chronic renal failure. The study protocol was approved by the institutional review boards of the Johns Hopkins University and Loayza Hospital. All study participants gave informed consent.

Patients who agreed to participate in the study answered a brief questionnaire and underwent physical examination. The medical records were reviewed. A tuberculin skin test (TST) (5 tuberculin units, Connaught, Swiftwater, PA) was administered and was read after 48 to 72 hours. The TST was considered positive if the area of induration measured ≥10 mm both vertically and horizontally. At least one sputum specimen >1 mL in volume was obtained; whenever possible, additional sputum specimens were obtained on consecutive days.

Laboratory Testing for TB

Acid-fast Bacilli Smear Microscopy

All samples were digested and concentrated by the standard N-acetyl-L-cysteine NaOH-Na citrate method for processing mycobacterial specimens (8). Ziehl-Neelsen and Auramine staining were performed by standard techniques (8).

Cultures

Mycobacterial growth indicator tubes (Becton Dickinson, Sparks, MD) containing both 10% OADC (oleic acid, albumin, dextrose, and catalase) (Becton Dickinson, Sparks, MD), and 100 µL of PANTA Antimicrobic Supplement (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, and Azlocillin) (Becton Dickinson) were injected with 500 µL of decontaminated sputum sample according to the manufacturer's specifications. Löwenstein-Jensen slants (Difco, Detroit, MI) and Middlebrook 7H11 medium plates (Difco, Detroit, MI) were injected with 250 µL of decontaminated sample. Tubes were incubated at 37°C and examined for mycobacterial growth at least weekly for up to 6 weeks with a 365-nm UV transilluminator. Löwenstein-Jensen slants and micro-agar 7H11 plates were incubated at 37°C with and without 5% CO_2 and examined by light microscopy for mycobacterial growth at least weekly for 2 to 8 weeks after injection (8). Criteria for positive mycobacterial growth have been previously described by the Centers for Disease Control (9).

Sensitivity Testing

The microplate alamar blue assay was used to determine mycobacterial drug resistance (10). Bacterial suspensions were prepared from colonies grown on Middlebrook 7H11 agar. Samples of the bacterial suspension (20 µL) were grown in 96-well plates containing serial dilutions of anti-TB drugs (isoniazid, rifampin, ethambutol, streptomycin, capreomycin, ciprofloxacin) until control wells tested positive for mycobacterial growth, usually in 5 to 6 days. Alamar blue reagent was then added to each well, and mycobacterial growth was identified by a change in media color from blue to pink. MIC was defined as the lowest drug concentration at which no blue-to-pink color change was observed. MICs for the panel of six anti-TB drugs were determined for each isolate.

Data Analysis

Patients were included in the study if they completed the questionnaire, had a physical examination, and provided one adequate sputum specimen. A patient was considered to have MDRTB if the sputum exhibited growth in media containing both isoniazid and rifampin. HIV tests were not performed as part of this study, but HIV test results were available for some patients.

All data were entered twice, and the two databases were compared to eliminate data entry errors. Data were analyzed with SPSS version 7.5 (SPSS Inc., Chicago, IL) and Epi Info version 6.0 (CDC, Atlanta, GA). The chi-square and Fisher's exact tests were used to measure strengths of association for categorical variables. The Wilcoxon 2-sample test was used to compare continuous variables.

Results

From January to December 1997, 250 (78%) of 319 patients admitted to the ward had a completed questionnaire and physical examination and at least one adequate sputum specimen. Forty patients (16%) had sputum cultures positive for *Mycobacterium tuberculosis*, and 26 of these had positive sputum smears. One patient

had a positive smear but a negative culture. Only three patients had a diagnosis of HIV infection; none of the three had a positive sputum specimen. Of the 69 ward patients who declined to participate or were unable to provide an adequate sputum specimen, 4 (6%) had been admitted with a diagnosis of suspected TB. If we assume all excluded patients to be negative for TB, the minimum estimated TB prevalence on the ward was 13%.

Patients with a cough of any duration, a cough that lasted >2 weeks, reported weight loss, hemoptysis, or a family history of TB were more likely to have sputum cultures positive for TB (Table 1). Anorexia was associated with a lower likelihood of TB. Because of logistic constraints, we were able to place and read a TST at 48 to 72 hours only on a subset of patients. Of the 67 patients with TST results, a positive reading was observed in 11 (55%) of 20 culture-positive patients compared with 10 (21%) of 47 patients without TB (p=0.007). Among culture-positive patients, those with a positive TST response were younger than those with a negative reading (median 23 years of age[range 19-66] vs. 47 years [range 25-88], p=0.02 by Wilcoxon 2-sample test). The socioeconomic status of patients with and without TB was similar.

Table 1. Female patients admitted to a general medicine ward of a hospital, Lima, Peru

	Mycobacterium tuberculosis culture results	
Characteristic	Positive N=40, n (%)	Negative[a] N=209, n (%)
Median age (range)	43 (18-96)	46 (14-92)
Cough	35 (88)[b]	125 (60)[b]
Cough for ≥ 2 weeks	25 (63)[b]	64 (31)[b]
Weight loss	33 (83)[b]	122 (58)[b]
Hemoptysis	12 (30)[c]	29 (14)[c]
Anorexia	22 (55)[c]	149 (71)[c]
Fever	24 (60)	108 (51)
Dyspnea	22 (55)	107 (51)
TST positive[d]	11 (55)[c]	10 (21)[c]
BCG scar	28 (70)	150 (71)
History of BCG vaccination	29 (73)	152 (72)
Family history of TB	12 (30)[c]	32 (15)[c]
Prior history of TB	9 (23)	34 (16)
Socioeconomic indicators		
Electricity in home	36 (90)	196 (93)
Piped water	32 (80)	180 (86)
Able to read and write	31 (78)	159 (76)

[a]One patient who was smear positive but culture negative was excluded from the analysis.
[b]P value < 0.01 by Mantel-Haenzsel chi-square test.
[c]P value < 0.05 by Mantel-Haenzsel chi-square test.
[d]A total of 67 patients, 20 *M. tuberculosis* culture-positive and 47 *M. tuberculosis* culture-negative, had tuberculin skin tests (TST).

Of the 181 patients who reported past BCG immunization, 178 (98%) had a scar. No vaccine scars were observed among the 68 persons who reported no history of BCG immunization. However, having a BCG scar was not associated with any apparent protective effect (Table 1). The presence of a BCG scar was not associated with a positive TST, even when TB culture positive patients were excluded (p=0.7).

Of 40 patients with at least one positive sputum culture, 23 (58%) had strains resistant to at least isoniazid, 8 (20%) to rifampin, 4 (10%) to ethambutol, and 1 (3%) to streptomycin. None were resistant to ciprofloxacin or capreomycin. Eight patients (20%) had TB resistant to both isoniazid and rifampin and were classified as having MDRTB. All 8 patients with resistance to rifampin also had resistance to isoniazid, and 15 patients had strains resistant to isoniazid but not to rifampin. Of the eight strains resistant to both isoniazid and rifampin, one was also resistant to ethambutol, one to streptomycin, and one to both ethambutol and streptomycin. Of 8 patients with MDRTB, 3 had a previous history of TB treatment.

Culture-positive patients for whom TB was the admitting diagnosis differed from those in whom TB was not suspected at the time of admission (Table 2). Patients whose TB had not been suspected were older and less likely to have the classic findings of cough, hemoptysis, weight loss, and prior personal or family history of TB. Patients whose TB had not been suspected at the time of admission were less likely to have a positive sputum smear, but this difference did not reach statistical significance (p=0.16 by Fisher's exact test). However, patients whose TB had not been suspected were significantly more likely to have MDRTB. Six (75%) of 8 patients with MDRTB were not suspected to have TB on admission; 3 (50%) of these six were also smear positive. Admitting diagnoses among culture-positive patients whose TB had not been suspected on admission included two patients with diabetes mellitus, one with systemic lupus erythematosus, and one with a lung lesion thought to be a hydatid cyst.

Table 2. *Mycobacterium tuberculosis* culture-positive patients, by admission diagnosis, Lima, Peru

Characteristic	*M. tuberculosis* culture-positive patients Suspected TB N=27, n (%)	No suspected TB N=13, n (%)
Median age (range)	27 (18-87)[a]	58 (22-96)[a]
Cough	27 (100)[b]	8 (62)[b]
Cough for $\geq$ 2 weeks	20 (74)[c]	5 (39)[c]
Weight loss	25 (93)[c]	8 (62)[c]
Hemoptysis	10 (37)	2 (15)
Fever	16 (59)	8 (62)
Anorexia	13 (48)	9 (69)
Dyspnea	17 (63)	5 (39)
Prior history of TB	8 (30)	1 (8)
Family history of TB	10 (37)	2 (15)
Smear positive	20 (74)	6 (46)
MDRTB	2 (7)[c]	6 (46)[c]
MDRTB and smear positive	2 (7)	3 (23)

[a]p value < 0.05 by Wilcoxon 2-sample test.
[b]p value < 0.01 by Fisher's exact 2-tailed test.
[c]p value < 0.05 by Fisher's exact 2-tailed test.
MDRTB = Multidrug-resistant tuberculosis.

Conclusions

The overall prevalence of TB among our study patients was high: at least 13% of all patients admitted to this general medicine ward had active TB. Two-thirds of TB patients were smear positive and therefore highly infectious, one-fifth had multidrug-resistant strains, and 75% of the patients with MDRTB had not been suspected of having TB when they entered the hospital. As in most Latin American hospitals, no masks or other respiratory devices were used to prevent spread in this hospital, even when the patient was known to be smear positive and highly infectious.

Nosocomial outbreaks of MDRTB in the United States in the 1980s and early 1990s heightened enforcement of stringent hospital control measures (11), leading to measurable decreases in TST conversion rates among hospital staff (12). Although the rate of TB in Peru is approximately 20 times higher than that of New York City (13), no concerted effort has been made to improve TB control measures in Peruvian hospitals.

The spread of MDRTB threatens control efforts (14). The fact that the majority of our patients with MDRTB had no history of past treatment of TB implies that person-to-person transmission of multidrug resistant strains occurs in Peru. Our data suggest that hospital wards may be one of the sites of transmission.

In developing countries where resources are limited, TB control programs focus on identification and treatment of infectious cases (15). Although treatment is clearly an important component of control, person-to-person spread of resistant strains makes isolation a high priority for preventing transmission. TST testing was not useful in identifying the group in need of screening. Anergy, which was common among culture-positive TB cases, was associated statistically with older median age and was perhaps related to concurrent systemic illness and poor nutritional status among hospitalized patients.

Although Peru has implemented an effective community-based TB control program, hospital control has not been a focus. Control measures such as isolation and respiratory precautions, stringently enforced in the past, were relaxed worldwide after the advent of inexpensive, effective anti-TB medications. After 50 years of selective drug pressure, the outbreak of MDRTB in New York City (5) dramatically highlighted the consequences of lapses in infection control.

Our data show that in countries or locales with a known high prevalence of TB, hospitals should screen all patients with respiratory symptoms by sputum smear within 12 hours of admission to hospital. Those found to be smear-positive should be placed in respiratory isolation, apart from TB-negative patients, until the smear becomes negative. Hospital personnel should observe respiratory precautions in caring for these patients. A system of rapid culture diagnosis and susceptibility testing should be implemented, allowing the presumptive diagnosis of MDRTB within 2 weeks (16). In combination, admission screening for TB, re-implementation of effective hospital respiratory control, and rapid TB diagnosis can substantially decrease the transmission of TB, especially MDRTB, in countries like Peru.

Acknowledgments

We thank R. Black, D. Berg, and K. Laserson for helpful comments and J.B. Phu and D. Sara for their assistance.

This study was supported in part by NIH grant number U01-AI35894-03, World AIDS Federation grant number 94.093, Fogarty, FIRCA TW00611 and ITREID and the anonymous RG-ER fund.

Mr. Willingham, a fourth-year medical student at the University of Maryland, performed this study after he completed his Masters in Public Health at the Johns Hopkins School of Public Health. His research interests focus on tuberculosis, infectious diseases, and public health.

References

1. Dolin PJ, Raviglione MC, Kochi A. Global tuberculosis incidence and mortality during 1990-2000. Bull World Health Organ 1994; 72:213-20.
2. Sanghavi DM, Gilman RH, Lescano-Guevara AG, Checkley W, Cabrera LZ, Cardenas V. Hyperendemic pulmonary tuberculosis in a Peruvian shantytown. Am J Epidemiol 1998;148:384-9.
3. Raviglione MC, Dye C, Schmidt S, Kochi A. Assessment of worldwide tuberculosis control. Lancet 1997;350:624-9.
4. Pablos-Mendez A, Raviglione MC, Laszlo A, Binkin N, Rieder HL, Bustreo F, et al. Global surveillance for antituberculosis-drug resistance, 1994-1997. N Engl J Med 1998;338:1641-9.
5. Edlin BR, Tokars JI, Grieco MH, Crawford JT, Williams J, Sordillo EM, et al. An outbreak of multidrug resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. N Engl J Med 1992; 326:1514-21.
6. Beck-Sague C, Dooley SW, Hutton MD, Otten J, Breeden A, Crawford JT, et al. Hospital outbreak of multidrug resistant *Mycobacterium tuberculosis* infections. JAMA 1992;268:1280-6.
7. Kritski AL, Marques MJ, Rabahi MF, Vieira MA, Werneck-Barroso E, Carvalho CE, et al. Transmission of tuberculosis to close contacts of patients with multidrug-resistant tuberculosis. Am J Respir Crit Care Med 1996;153:331-5.
8. Welch DF, Guruswamy AP, Sides SJ, Shaw CH, Gilchrist MJR. Timely culture for *Mycobacteria* which utilizes a microcolony method. J Clin Microbiol 1993;31:2178-84.
9. Kent BD, Kubica GP. Public health mycobacteriology: A guide for the level III laboratory. Atlanta: Department of Health and Human Services, Centers for Disease Control, 1985;36-9, 47-69,185-7.
10. Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, et al. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 1998;36:362-6.
11. Stricof RL, DiFerdinando GT, Osten WM, Novick LF. Tuberculosis control in New York City hospitals. Am J Infect Control 1998;26:270-6.
12. Bangsberg DR, Crowley K, Moss A, Dobkin JF, McGregor C, Neu HC. Reduction in tuberculin skin-test conversions among medical house staff associated with improved tuberculosis infection control practices. Infect Control Hosp Epidemiol 1997;18:566-70.
13. Hoyos C, Izquierdo G, Piscoya G, Romero M, Saldías J. [Incidence of infective diseases at an internal medicine service]. Rev Gastroenterol Peru 1991;11:171-5.
14. Centers for Disease Control and Prevention. Multi-drug-resistant tuberculosis outbreak on an HIV ward – Madrid, Spain, 1991-1995. MMWR Morb Mortal Wkly Rep 1996;45:330-3.
15. Enarson DA, Grosset J, Mwinga A, Hershfield ES, O'Brien R, Cole S, et al. The challenge of tuberculosis: statement on global control and prevention. Lancet 1995;346:809-19.
16. Caviedes L, Lee TS, Gilman RH, Sheen P, Speelman E, Lee EH, et al. Rapid, efficient detection and drug susceptibility testing of *Mycobacterium tuberculosis* in sputum by microscopic observation of broth cultures. J Clin Microbiol 2000;38:1203-8.

Outbreak of West Nile Virus Infection, Volgograd Region, Russia, 1999

Alexander E. Platonov,* German A. Shipulin,* Olga Yu. Shipulina,* Elena N. Tyutyunnik,† Tatyana I. Frolochkina,‡ Robert S. Lanciotti,§ Svetlana Yazyshina,¶ Olga V. Platonova, * Igor L. Obukhov,¶ Alexander N. Zhukov, Yury Ya. Vengerov,† and Valenin I. Pokrovskii***

*Central Institute of Epidemiology, Moscow, Russia; †Moscow State University for Medicine and Dentistry, Russia; ‡Ministry of Public Health, Moscow, Russia; §Centers for Disease Control and Prevention, Fort Collins, Colorado, USA; ¶Russian State Institute for Control of Veterinary Products, Moscow, Russia; **Center of Sanitary and Epidemic Control for Volgograd Region, Volgograd, Russia

From July 25 to October 1, 1999, 826 patients were admitted to Volgograd Region, Russia, hospitals with acute aseptic meningoencephalitis, meningitis, or fever consistent with arboviral infection. Of 84 cases of meningoencephalitis, 40 were fatal. Fourteen brain specimens were positive in reverse transcriptase-polymerase chain reaction assays, confirming the presence of West Nile/Kunjin virus.

West Nile (WN) virus is a member of the Japanese encephalitis (JE) antigenic complex of the genus Flavivirus, family Flaviviridae. Mosquito-borne WN virus fever is endemic in Africa, the Middle East, and Southwest Asia. The antigenically and genetically related Kunjin virus is a WN virus counterpart in Australia and Southeast Asia and has recently been taxonomically classified as a subtype of WN virus. Until recently, WN virus infection in humans was considered a relatively mild, influenzalike disease with full recovery, although occasionally (<15% of cases) acute aseptic meningitis or encephalitis occurred (1). No large outbreak of WN virus fever was reported in Europe until August and September 1996, when more than 500 clinical cases were observed in Romania (Bucharest region), with high rates of neurologic disorders and death (up to 10%) (2). WN virus had never been detected in the Western Hemisphere until August 1999, when an outbreak of human WN encephalitis in New York City (56 confirmed cases, 7 deaths) coincided with unusual deaths in crows and exotic birds (3-5).

Address for correspondence: A.E. Platonov, Central Institute of Epidemiology, Novogireevskaya Str. 3A, Moscow 111123, Russia; Fax: 7-095-305-5423; e-mail: plt@online.ru.

The Study

In August and September 1999, an outbreak of acute viral infection consistent with arboviral infection occurred in the Volgograd Region, Russia. Epidemiologic and clinical data were collected and analyzed in the Center of Sanitary and Epidemic Control for the Volgograd Region in collaboration with the Commission of the Russian Ministry of Public Health. From July 25 to October 1, 826 patients were admitted to area hospitals with the clinical diagnosis of acute aseptic meningoencephalitis (code A86, ICD-10; 84 patients), acute aseptic meningitis (code A87.9; 308 patients), or acute viral infection with fever (code B34.9; 347 patients). Serum samples from 318 patients were tested for WN virus antibody by immunoglobulin (Ig)M-capture enzyme-linked immunosorbent assay (ELISA) and indirect IgG ELISA (2,6); 183 (58%) samples demonstrated a level of anti-WN virus IgM indicative of acute infection. This proportion was approximately the same in patients with aseptic meningoencephalitis, aseptic meningitis, and acute fever. These 183 cases were considered serologically confirmed WN virus cases; all 826 cases were considered clinically compatible WN virus cases. The total number of suspected overt human WN virus cases was estimated to be 480.

Volgograd City (population 1 million) is located on the west bank of the Great Volga River (latitude 48°N, longitude 44°E) in the Russian steppe; Volzskii City (population 300,000) is on the opposite bank. Approximately 65% and 30% of WN virus cases were from Volgograd and Volzskii, respectively; the rest occurred in the rural region around Volgograd, near the Volga River or its tributaries. The male:female infection ratio was 1:1. The incidence of infection was age specific; more than 50% of patients were ≥50 years of age and less than 15% were ≤15 years. The epidemic peaked between August 21 and August 25, waning with the onset of cooler temperatures in late September. Figures were similar for serologically confirmed and clinically probable WN virus cases.

The clinical characteristics of the Volgograd WN virus epidemic differed somewhat from those of previous outbreaks (1). In Volgograd, the disease was generally more severe, with a higher than normal case-fatality rate. The central nervous system was usually involved, and acute aseptic meningitis or encephalitis was frequently observed. Rash and conjunctivitis were rarely observed. Abdominal pain, diarrhea, respiratory symptoms, and lymphadenopathy were rare or absent. As in other WN virus epidemics, clinical features included abrupt onset of disease, asthenia, high fever (up to 39°C-40°C), headache, and vomiting.

Of the 84 cases of acute aseptic meningoencephalitis, 40 were fatal (7). Autopsies of laboratory-confirmed WN meningoencephalitis cases revealed perivascular hemorrhages, ectasis of ventriculi of the brain, foci of encephalomalacia, dislocation of the brain trunk (30% of cases), and hydropericarditis with flabbiness of the cardiac muscle. Microscopy findings included signs of focal encephalitis and vasculitis, lymphocytic perivascular inflammatory infiltration, profound degenerative and necrobiotic changes of ganglion cells in the cerebral cortex, and signs of brain edema, as well as parenchymal myocarditis (stromal edema, degeneration of myocytes, foci of myolysis, and fragmentation of myofibrils). Thirty (75%) of the patients who died were >60 years of age.

We have developed reverse transcriptase-polymerase chain reaction (RT-PCR) assays specific for WN/Kunjin genome and a consensus assay for the detection of all flavivirus genomes (5,8, 9). Two pairs of oligonucleotide primers (WN11/WN2, WN1/WN2) were designed to hybridize to a relatively conserved region within the envelope (E) gene of WN/Kunjin viruses. The expected amplification product was 222 base pairs long (WN1, 5'- AGG, GGC, CAC, CCA, GGC, TGG, AAG, ATT, CA- 3'; WN11, 5'-TGG, GGC, CAC, TCA, GGC, AGG, GAG, ATT, CA-3'; WN2, 5'-CAC, GTG, GTG, CTT, CCA, GCA, CTG, CTC, CA-3'). Another pair of primers, FLV1/FLV2, was designed to hybridize to conserved regions within the RNA replicase (NS5) gene of a wide variety of flaviviruses, amplifying nearly a 220-bp fragment (FLV1, 5'-GGI, AGC, AGI, GCC, ATI, TGG, T(A/T)C, ATG, TGG - 3'; FLV-2, 5'-C(G/T)I, GTG, TCC, CAI, CCI, GCI, GTG, TCA, TC-3').

Brain tissue samples taken at autopsy from 14 patients with meningoencephalitis were subjected to RT-PCR with primer pairs WN11/WN2, WN1/WN2, and FLV1/FLV2. The samples and corresponding viral RNA/cDNA preparations are designated below as Volgograd-1999-1, Volgograd-1999-2, and the like. Two WN virus strains isolated in 1967-1970 in the Republic of Azerbaijan (former Soviet Union) and the prototype JE strain isolated in Tokyo were used as control templates in the RT-PCR assay. All 14 brain samples were strongly positive in the RT-PCR assays with WN11/WN2, WN1/WN2, and FLV1/FLV2, confirming the presence of WN/Kunjin sequences. The WN-Azerbaijan-1967 and WN-Azerbaijan-1970 control RNA preparations were positive only with WN11/WN2 and FLV1/FLV2 primer pairs, indicating that some differences in the corresponding region of the E gene from the old and new “Russian” WN virus strains were likely. As expected, the JE-Tokyo-1935 RNA preparation was negative in the WN-specific RT-PCR assay, but positive with FLV1/FLV2 primers.

The amplification products obtained from the E and NS5 genes from seven patients (#1, 3, 7, 11, 12, 13, 14) and from the reference WN-Azerbaijan-1967 and WN-Azerbaijan-1970 strains were subjected to DNA sequencing. The sequences obtained from the Volgograd patients were identical, suggesting infection with a single WN virus strain.

The E sequences of the Volgograd and Azerbaijan WN viruses were aligned with each other and with 38 other WN/Kunjin strains, by using CLUSTAL W alignment software. The NS5 sequences of the Volgograd and Azerbaijan WN viruses were aligned with 16 other flavivirus

strains by CLUSTAL W. The high level of sequence similarity confirmed the WN virus source of the Volgograd cases (Table). Phylogenetic trees of WN/Kunjin viruses and all flaviviruses derived from E and NS5 gene sequences (5,8-10) have been previously described. We used smaller gene fragments, 165 bases of E gene and 147 bases of NS5 gene; however, the deduced phylogenetic trees were practically the same. Therefore, we limited the analysis to the most similar strains, including the epidemiologically important WN-New-York-1999 and WN-Romania-1996 strains, and some representatives of other taxonomic subdivisions. For comparison, the designation of strains coincides with the designation in publications where the additional details of strain history are given (5,10).

The Volgograd and old Azerbaijan WN virus strains clearly belonged to "lineage 1" of WN virus isolates (5,9-10). Within lineage 1, the Volgograd patient strains were most closely related to the current Kenya and Senegal strains and the Romanian mosquito isolate (the identical E gene fragment of 165 nucleotides [nt]). The Azerbaijan isolates were more closely related to

Table. Percentage identity of nucleotide sequence for E and NS5 gene fragments among West Nile/Kunjin viruses

Virus strain	WN-Romania-1996M	WN-Kenya-1998 and WN-Senegal-1993	WN-New York-1999F and WN-New York-1999H	WN-Azerbaijan-1967	WN-Azerbaijan-1970	WN-Romania-1996H	WN-Egypt-1951	Kunjin-Aust-1960	WN-Nigeria (Wengler)
WN-Volgograd-1999-1 and WN-Volgograd-1999-11*	100	100 96.6	98.2 94.6	93.9 95.2	93.9	93.9 92.5	92.3 85.0	86.7 78.2	74.5
Romania-1996M		100	98.2 96.6	93.9 94.6	93.9 95.2	93.9	92.3 92.5	86.7 85.0	74.5 78.2
WN-Kenya-1998 and WN-Senegal-1993			98.2	93.9	93.9	93.9	92.3	86.7	74.5
WN-New York-1999F and WN-New York-1999H				95.8 95.2	95.8 94.6	95.8	94.5 93.2	87.3 85.7	75.8 78.9
WN-Azerbaijan-1967					100 99.3	100	97.6 98.0	88.5 87.8	75.8 78.2
WN-Azerbaijan-1970						100	97.6 97.3	88.5 88.4	75.8 78.2
WN-Romania-1996H							97.6	88.5	75.8
WN-Egypt-1951								87.9 87.1	75.8 76.2
Kunjin-Aust-1960									75.1 72.8
WN-Nigeria (Wengler)									

*First lines in a row = percentage identity of nucleotide sequence for E gene fragment; second lines in a row = percentage identity of nucleotide sequence for NS5 gene fragment (if available). The strains with identical sequences of E and NS5 fragments are placed in the same row or column.

WN-Egypt-1951 strain and the Romanian human isolate (Figure). The New York-1999 isolate differed from the Volgograd isolates in 3 nt positions and from the Azerbaijan isolates in 7 nt positions. All 10 polymorphic sites in sequences of Volgograd, New York, Azerbaijan, and Romania strains were in the third codon position, producing silent mutations. WN-Volgograd-1 differed from WN-Egypt-1951 in 1 amino acid (aa) (Leu vs. Trp), from Kunjin in 1 aa (Asn vs. Ser) and from WN-Nigeria in 5 aa within the E fragment of 55 aa.

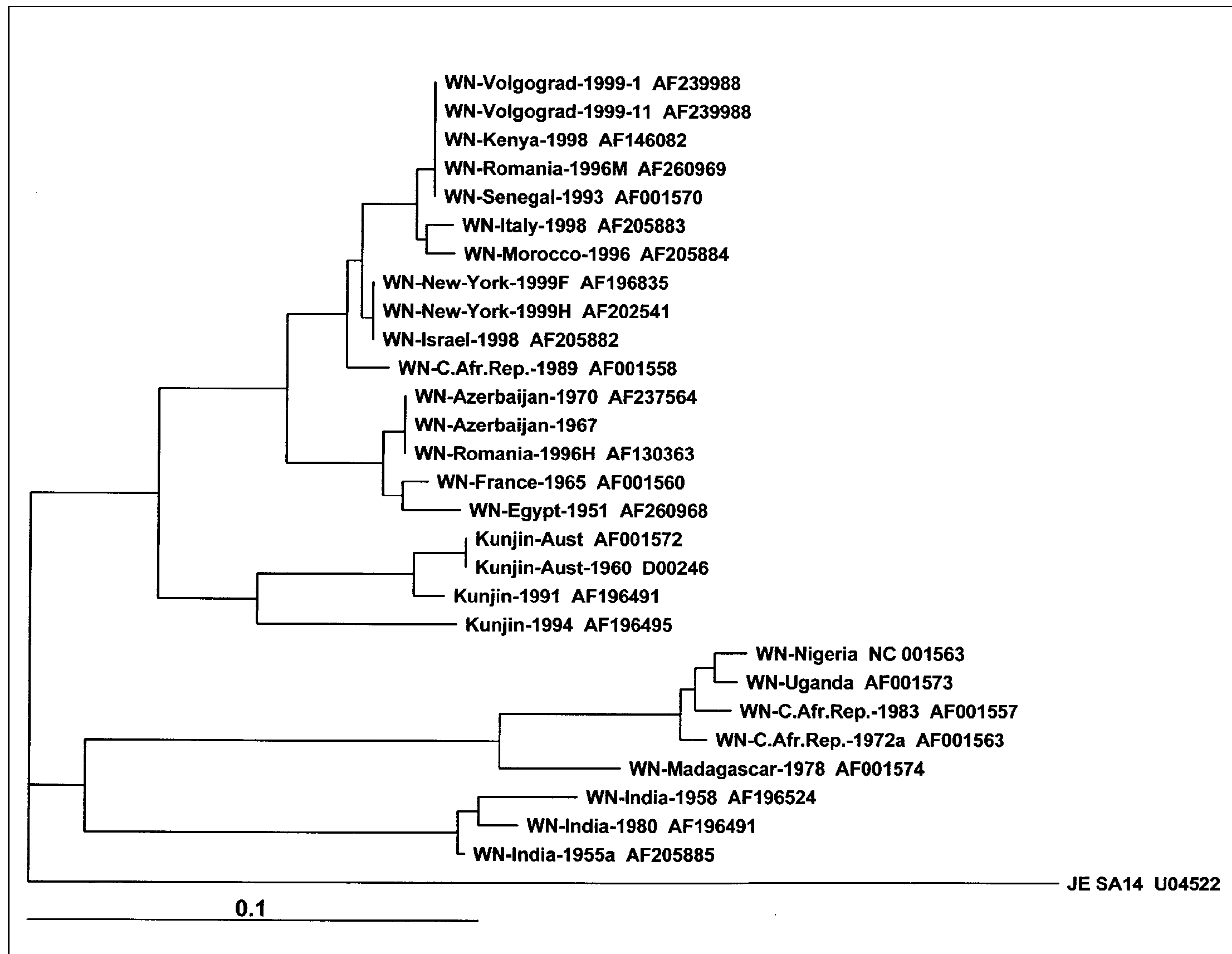


Figure. Phylogenetic trees based on nucleic sequence data of E-glycoprotein gene fragment of 165 bp. The trees were constructed with the program CLUSTAL by using the neighbor joining method of Saitou and Nei with bootstrapping. Tree is rooted by using Japanese encephalitis sequence as an outgroup. The designation of isolates corresponds to that in publications (5,10), where details of isolate history are given. Alignments used for analysis are available upon request from the authors.

[a]WN virus strains used in phylogenetic analysis. WN-Volgograd-1999-1 = human brain tissue from female patient, Volgograd, Russia, GenBank accession # AF239988 (E gene fragment) and #AF239990 (NS5 gene fragment); WN-Kenya-1998 = strain KN3829 isolated from *Culex univittatus*, # AF146082; WN-Senegal-1993 = strain SEN-ArD93548 isolated from *Culex neavei*, # AF001570; WN-Romania-1996M = strain RO97-50 isolated from *Culex pipiens* pool, # AF260969; WN-New-York-1999F = strain NY99-flamingo382-99 isolated from Bronx Zoo flamingo 1999, # AF196835; WN-New-York-1999H = strain HNY1999 isolated from total human brain RNA, # AF202541; WN-Azerbaijan-1967 = isolated from a bird, # AF241822 (NS5 gene fragment); WN-Azerbaijan-1970 = strain A-72 isolated from a tick *Ornitodorus coniceps,* # AF241821 (NS5 gene fragment) and # AF237564 (E gene fragment); WN-Romania-1996H = strain RO96-1030 isolated from human CSF, # AF130363; WN-Egypt-1951 = strain Eg101, #AF260968; Kunjin-Aust-1960 = Kunjin virus MRM61C, # D00246; WN-Nigeria = West Nile virus, # M12294 and #NC_001563.

Although a few sequences of WN virus NS5 gene were available, the data confirmed that the WN virus strains of lineage 1 were more close to each other (the differences in 0-11 nt of 147) than to Kunjin, and especially distant from WN-Nigeria (Wengler) strain (Table). Again, all 10 polymorphic sites in sequences of lineage 1 strains corresponded to the silent mutations only. WN-Volgograd-1 differed from Kunjin in 1 aa (Lys vs. Arg) and from WN-Nigeria in 3 aa (Arg vs. Lys, Arg vs. Lys, and Ile vs. Val) but differed, for example, from Saint Louis (AF013416) in 11 aa within the NS5 fragment of 48 aa. The isolation of WN virus from one of our clinical brain samples, Volgograd-1999-4, will make complete genome sequencing and further virologic investigations possible (11).

Conclusions

Our data, together with those of previous publications, document several outbreaks of emerging WN virus infection in regions where this disease was not found or was rarely found. (There were a few isolations of WN virus in Romania and the former Soviet Union before 1996-99.) Some isolates demonstrate a high degree of similarity (New York-1999 and Israel-1998; Volgograd-1999, Romania-1996-mosquito isolate, Kenya-1998, and Senegal-1993; Azerbaijan-1967 and Romania-1996-human isolate). Moreover, the last three large outbreaks were caused by genetically similar strains (WN-Romania-1996, WN-New York-1999, and WN-Volgograd-1999), indicating the wide circulation and emergence of potentially epidemic strains of WN virus. All three cities, Bucharest, New York, and Volgograd, are located near large bodies of water and on bird migration pathways and all had unusually dry summers the year of the outbreak (12). Some clinical characteristics of the recent WN virus epidemics were unexpected, such as the high rate of neurologic disorders and death. These unusual characteristics may be due to the expansion of new pathogenic WN virus strain(s) or to the peculiarities of the human host response.

Acknowledgments

We thank DK Lvov, VL Gromashevskii, AM Butenko, S. Ya. Gaidamovitch, O Vyshemirskii, and VF Larichev for providing the reference WN virus strains and helpful discussions; DJ Gubler, J LeDuc, and P Henry for arranging research collaboration between the Central Institute of Epidemiology, Moscow, and the Centers for Disease Control and Prevention; and NV Rusakova, EM Krasnova, VA Petrov, and AM Alyushin for providing human specimens for testing, and epidemiologic and clinical information for analysis.

Dr. Platonov is head of a laboratory at the Central Institute of Epidemiology, Russia. His scientific interests include the epidemiology, diagnosis, and pathogenesis of bacterial and viral meningitis.

References

1. Hubálek Z, Halouzka J. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg Infect Dis 1999;5:643-50.
2. Tsai TF, Popovici F, Cernescu C, Campbell GL, Nedelcu NI. West Nile encephalitis epidemic in southeastern Romania. Lancet 1998;352:767-71.
3. CDC. Update: West Nile virus encephalitis—New York, 1999. Morb Mortal Wkly Rep 1999;48:944-6.
4. Anderson JF, Andreadis TG, Vossbrinck CR, Tirrell S, Wakem EM, French RA, et al. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science 1999;286:2331-3.
5. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern U.S. Science 1999;286:2333-7.
6. Lvov DR, Butenko AM, Gaidamovitch SY, Larichev VPh, Leschinskaya EV, Zhukov AN, et al. Epidemic outbreak of meningitis and meningoencephalitis in Krasnodar territory and Volgograd region provoked by of West Nile fever virus. Vopr Virusol 2000;45:37-8.(In Russian).
7. Vengerov YuYa, Frolochkina TI, Zhukov AN, Shipulin GA, Shipulina OYu, et al. West Nile virus infection as clinical and epidemiological problem. Epidemiology and Infectious Diseases 2000;4:27-31.(In Russian).
8. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus Flavivirus. J Virol 1998;72:73-83.
9. Berthet FX, Zeller HG, Drouet MT, Rauzier J, Digoutte JP, Deubel V. Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. J Gen Virol 1997;8(Pt 9):2293-7.
10. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, et al. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. Am J Trop Med Hyg 1999;61:600-11.
11. Lvov DR, Butenko AM, Gromashevsky VI, Larichev VPh, Gaidamovitch SYa, Vyshemirsky OI, et al. Isolation of two strains of West Nile virus during an outbreak in Southern Russia, 1999. Emerg Infect Dis 2000;6:373-6.
12. Han LL, Popovici F, Alexander Jr JP, Laurentia V, Tengelsen LA, Cernescu C, et al. Risk factors for West Nile virus infection and meningoencephalitis, Romania, 1996. J Infect Dis 1999;179:230-3.

Rapid Identification of *Corynebacterium diphtheriae* Clonal Group Associated with Diphtheria Epidemic, Russian Federation

Svetlana Kombarova,* Chung Kim,† Viatcheslav Melnikov,* Michael Reeves,† Olja Borisova,* Izabella Mazurova,* and Tanja Popovic*†

*Gabrichevsky Institute of Epidemiology and Microbiology, Moscow, Russia;
†Centers for Disease Control and Prevention, Atlanta, Georgia, USA

We used 199 *Corynebacterium diphtheriae* isolated from 1995 to 1997 in Russia to evaluate the ability of random amplified polymorphic DNA (RAPD) to identify the unique clonal group that emerged there in 1990. Our data show that RAPD can reliably, reproducibly, and rapidly screen a large number of strains to identify the epidemic clonal group.

Molecular subtyping, primarily by multilocus enzyme electrophoresis (MEE) and ribotyping, has identified substantial genetic diversity within the *Corynebacterium diphtheriae* species, leading to the identification of a unique clonal group that emerged in Russia in 1990 at the beginning of the current diphtheria epidemic (1). Strains of this clonal group belong to a distinct electrophoretic type complex (ET8 complex) and are of ribotypes G1 and G4. Identification of this clonal group has permitted precise monitoring of the epidemic's growth and rapid detection of imported cases in neighboring and other European countries.

Use of traditional subtyping methods in monitoring the expansion of the epidemic clone has helped differentiate epidemic, endemic, and imported cases and has allowed timely preventive measures. Even as the epidemic declines (from more than 50,000 cases in 1995 to 1,436 cases in 1998), identifying organisms belonging to this epidemic clone in cases of suspected importation into locations where diphtheria is rarely encountered continues to provide valuable information. Since both ribotyping and MEE are time-consuming, taking 3-4 working days to produce results, rapid methods that could distinguish the predominant clone would improve epidemic surveillance and prevention measures.

Random amplified polymorphic DNA (RAPD) is a simple and rapid molecular subtyping method. Recently, Nakao et al. (2) optimized and standardized this assay for *C. diphtheriae* and showed that the discrimination level obtained by RAPD correlated well with that of ribotyping; each of 20-plus ribotyping patterns was associated with one or more distinct RAPD patterns. We compared these two techniques on a large number of *C. diphtheriae* Russian isolates from 1995 to 1997, focusing on the ability of RAPD to identify the isolates of the epidemic G1/4 clonal group.

The Study

All *C. diphtheriae* strains were collected by the Russian Federal Diphtheria Diagnosis Reference Laboratory. Of 199 isolates from different regions of Russia, 187 were isolated from 1995 to 1997; 12 were isolated during 1993 to 1994; 68 were recovered from clinically diagnosed diphtheria patients; and the remaining 131 isolates were obtained from carriers.

Identification, biotype, and toxigenicity determination were performed by standard

Address for correspondence: Dr. Tanja Popovic, Centers for Disease Control and Prevention, Mail Stop G34, 1600 Clifton Rd., Atlanta, GA 30333, USA; fax: 404-639-3172; e-mail: txp1@cdc.gov.

microbiologic methods (3,4). RAPD was performed by the Ready-To-Go RAPD Kit (Pharmacia Biotech, Piscataway, NJ)(2). RAPD type designations were adopted from those previously documented by Nakao et al. (2). Ribotyping was carried out as previously described (1) with some modifications (Table). MEE was performed as previously described (1). The genetic relatedness of the electrophoretic types (ETs) was illustrated as a dendrogram, generated by the average-linkage method of clustering the ETs (7) and by using an SAS macro program described by Jacobs (8).

Of the 199 *C. diphtheriae* isolates, 185 were biotype gravis, and 14 were biotype mitis. All isolates were toxigenic by the Elek assay. When assayed by RAPD using primers 3 and 4, the 199 isolates that were identical by primer 3 were also identical by primer 4, with the single exception of isolate B506. Of 185 isolates of the gravis biotype, 183 were the G1/4 RAPD type. Isolate B325 had a G4v pattern, and isolate B506 had the G1/4 pattern only by primer 4. When primer 3 was used on this isolate, a different pattern was observed.

The RAPD patterns of the 14 isolates of the mitis biotype were distributed into three groups. The first group included three isolates of the G1/4 RAPD type (B294, B399, B400). The second group included 10 isolates that had RAPD patterns types M1 and M1v. The third group included only one isolate (B306), which had a RAPD pattern completely different from any previously assayed strain.

Seventy-nine isolates were ribotyped. Of the 186 isolates with RAPD G1/4 patterns (183 gravis and 3 mitis), 66 were selected to be ribotyped for their geographic and temporal diversity. In addition, all non-G1/4 isolates were ribotyped. The ribotyping results correlated extremely well with the RAPD data (Table, Figure). With one exception, all isolates of the G1/4 RAPD type also had a G1 or G4 ribotype; 40 had the G1 ribotype, and 26 isolates possessed the G4 ribotype. In addition, the G4v ribotype was observed in the isolate with the G4v RAPD pattern. Isolate B306, which had an RAPD type not previously observed, also had a ribotype that did not resemble any previously established ribotypes. Five M1 and five M1v ribotypes were identified among the 10 M1/1v RAPD type isolates.

Twenty-nine isolates of RAPD types G1/4 and ribotypes G1 or G4 were analyzed by MEE. Among all isolates of this group, seven individual ETs, which clustered at a genetic distance of <0.12, were identified; all ETs were members of the previously defined ET8 complex. Only one to three enzyme differences from the ET8 complex were observed among the individual enzyme types (data not shown). The ET8 complex contains 27 ETs, which are related to each other at a genetic distance of 0.20 and have a maximum of four enzyme differences within the complex.

Table. Random amplified polymorphic DNA (RAPD) assay and ribotyping for 79 Russian *Corynebacterium diphtheriae* isolates[a,b]

Ribotype	Biotype	Number of strains	RAPD Primer 3	RAPD Primer 4
G1	G	38	G1/4	G1/4
	M	2	G1/4	G1/4
G4	G	25	G1/4	G1/4
	M	1	G1/4	G1/4
	G	1	New[c]	G1/4
G4v	G	1	G4v	G4v
M1	M	5	M1/1v	M1/1v
M1v	M	5	M1/1v	M1/1v
New	M	1	New	New

[a]G, biotype gravis; M, biotype mitis. Cultures were kept lyophilized at room temperature or were stored in defibrinated sheep blood and held at -70°C until needed. Before use, the strains were inoculated onto blood agar plates (trypticase soy agar with 5% sheep blood; Becton Dickinson, Cockeysville, MD) and were incubated at 37°C overnight.

[b]DNA for ribotyping was isolated by the universal isolation procedure (5). Hybridization of restricted DNA fragments was performed using a mixture of five digoxigenin-labeled oligonucleotide probes at 37°C for 4 hours as recently described by Regnault et al. (6). Posthybridization washes were also performed at 37°C in 2X SSC (1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) for 2x5 minutes and in 0.1X SSC, 0.1% SDS for 2x10 minutes. Detection was performed by using the DIG Wash and Block Buffer Set (Boehringer Mannheim Biochemicals, Indianapolis, IN), sheep anti-digoxigenin antibody conjugated with alkaline phosphatase, nitroblue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

[c]New = pattern had not been previously observed.

Conclusions

The *C. diphtheriae* epidemic clonal group associated with the recent diphtheria epidemic in the Russian Federation is characterized as being of ribotypes G1 and G4 and belonging to the ET8 complex. Detection of a unique epidemic clonal group has allowed continuous monitoring of the circulation of existing clones and rapid detection of new or unusual clones. The epidemic emphasized the need for continuous study of the

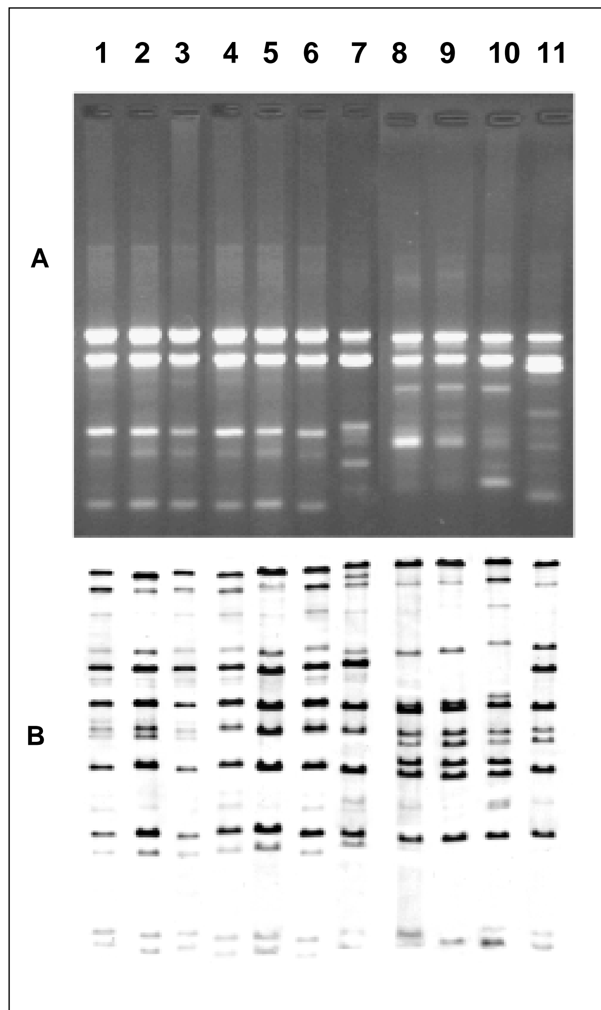


Figure. A) RAPD patterns of *Corynebacterium diphtheriae* isolated from 1995 to 1997. Lane 1, 1740 (strain #), gravis, G1/4 RAPD type strain. Lane 2, B327, gravis, G1/4 (RAPD type), 1997 (year of isolation). Lane 3, B400, mitis, G1/4, 1995. Lane 4, 490, gravis, G1/4 ribotyping type strain. Lane 5, B375, gravis, G1/4, 1995. Lane 6, B294, mitis, G1/4, 1996. Lane 7, B325, gravis, G4v, 1997. Lane 8, 860, mitis, M1/M1v RAPD type strain. Lane 9, B389, mitis, M1/M1v, 1995. Lane 10, B324, mitis, M1/M1v, 1997. Lane 11, B306, mitis, new RAPD pattern, 1997. B) Ribotyping patterns of *C. diphtheriae* isolated from 1995 to 1997. Lane 1, G4174 (strain #), gravis, G1 ribotyping type strain. Lane 2, B327, gravis, G1 (ribotype) 1997 (year of isolation). Lane 3, B400, mitis, G1, 1995. Lane 4, G4183, gravis, G4 ribotyping type strain. Lane 5, B375, gravis, G4, 1995. Lane 6, B294, mitis, G4, 1996. Lane 7, B325, gravis, G4v, 1997. Lane 8, G4212, mitis, M1 ribotyping type strain. Lane 9, B389, mitis, M1, 1995. Lane 10, B324, mitis, M1v, 1997. Lane 11, B306, mitis, new ribotype, 1997.

biologic properties of *C. diphtheriae*. Thus, a *Corynebacterium* ribotype database has been established, and substantial efforts are under way to standardize molecular subtyping approaches in diphtheria reference centers worldwide (9).

Given that ribotyping still takes several days to be completed, we evaluated the role RAPD might have as a rapid and reliable molecular subtyping tool by comparing its differentiation abilities to those of ribotyping; 199 *C. diphtheriae* isolates were analyzed, and RAPD was shown to be as discriminative as standard ribotyping. All but one isolate of ribotypes G1 or G4 were correctly identified as belonging to the G1/4 RAPD group by both primers. For comparative purposes, we analyzed a smaller number of isolates with M1 and M1v ribotypes; all of these isolates were also correctly identified as belonging to the M1/1v group by both RAPD primers. The two isolates that gave non-G1/4 or M1/1v ribotypes (B325 and B306) were obtained in the Asian part of Russia (Barnaul and Cheljabinsk, respectively).

Furthermore, of the 29 isolates that were analyzed by MEE, 7 closely related ETs (all members of the ET8 complex) were identified. These ETs differed from the predominant ET8 by one to three enzymes. MEE still provides a higher level of differentiation of the epidemic *C. diphtheriae* isolates (27 ETs) than ribotyping and RAPD (2 and 1 types, respectively). However, by all three methods, the isolates in our study were still clearly defined as belonging to the earlier described epidemic clonal group.

Our data unambiguously show that RAPD can be reliably and reproducibly used for rapidly screening strains of the predominant epidemic clonal group. Such rapid identification is extremely useful in investigations of potentially imported cases so that timely preventive measures can be implemented.

Dr. Kombarova is a microbiologist at the Gabrichevsky Institute of Epidemiology and Microbiology in Moscow, Russia. Her areas of expertise are isolation, identification, and molecular subtyping of *Corynebacterium diphtheriae*. She was extensively involved in identifying the epidemic clonal group associated with the diphtheria epidemic in Russia.

References

1. Popovic T, Kombarova S, Reeves M, Nakao H, Mazurova IK, Wharton M, et al. Molecular epidemiology of diphtheria in Russia, 1985-1994. J Infect Dis 1996;174:1064-72.
2. Nakao H, Popovic T. Use of random amplified polymorphic DNA for rapid molecular subtyping of *Corynebacterium diphtheriae*. Diagn Microbiol Infect Dis 1998;30:167-72.
3. Efstratiou A, Maple PA. WHO manual for the laboratory diagnosis of diphtheria. Geneva: World Health Organization; 1994. Reference no. ICP-EPI 038(C).
4. Mazurova IK, Melnikov V, Kombarova S. Manual for laboratory diagnostis of diphtheria infection. Moscow: Russian Federation State Committee on Sanitary Epidemiologic Surveillance;1995.
5. Graves LM, Swaminathan B. Universal bacterial DNA isolation procedure. In: Persing DH, Smith TR, Tenover FC, White TJ, editors. Diagnostic molecular microbiology. Washington: American Society for Microbiology; 1993. p. 617-21.
6. Regnault B, Grimont R, Grimont PAD. Universal ribotyping method using a chemically labeled oligonucleotide probe mixture. Res Microbiol 1997;146:649-59.
7. Selander RK, Daugant DA, Ochman H, Musser JM, Gilmore MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetic and systematics. Appl Environ Microbiol 1986;51:873-4.
8. Jacobs D. SAS/GRAPH software and numerical taxonomy. In: Proceedings of the 15th Annual Users Group International Conference. Cary (NC): SAS Institute; 1990. p. 1413-18.
9. Grimont PAD, Grimont F, Ruckly C. The *Corynebacterium diphtheriae* ribotype database. In: Program and abstracts of the 5th International Meeting of the European Laboratory Working Group on Diphtheria. London: Public Health Laboratory Service; 1998. p. 31.

Shigella spp. Surveillance in Indonesia: the Emergence or Reemergence of *S. dysenteriae*

Decy Subekti,* Buhari A. Oyofo,* Periska Tjaniadi,* Andrew L. Corwin,* Wita Larasati,* Maidy Putri,* Cyrus H. Simanjuntak,† Narain H. Punjabi,* Jufri Taslim,‡ Budhi Setiawan,§ Anak Agung G.S. Djelantik,¶ Luh Sriwati, Atiek Sumardiati,†† Eka Putra,‡‡ James R. Campbell,* and Murad Lesmana***

*U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia; †National Institute of Health Research and Development, Jakarta, Indonesia; ‡Municipal Health Service, North Jakarta, Indonesia; §Friendship Hospital, Jakarta, Indonesia; ¶Sanglah Hospital, Denpasar, Bali, Indonesia; **Wangaya Hospital, Denpasar, Bali, Indonesia; ††Regional Office of Health Ministry, Pontianak, Indonesia; and ‡‡Otorita Hospital, Batam, Indonesia

From June 1998 through November 1999, *Shigella* spp. were isolated in 5% of samples from 3,848 children and adults with severe diarrheal illness in hospitals throughout Indonesia. *S. dysenteriae* has reemerged in Bali, Kalimantan, and Batam and was detected in Jakarta after a hiatus of 15 years.

S*higella* spp. cause acute, debilitating diarrheal disease in humans (particularly young children) worldwide (1). In developing countries, where affected populations are immunologically compromised by poor nutrition and background infections, deaths attributed to shigellosis are common. Four *Shigella* species are recognized as pathogenic to humans: *S. sonnei, S. boydii, S. flexneri,* and *S. dysenteriae*. Both *S. sonnei* and *S. boydii* are usually associated with mild illness of short duration in which the stool may be watery or bloody (2). *S. flexneri* is generally more severe, lasts longer, and causes blood in stools. *S. dysenteriae*, particularly type 1, causes the most severe diarrheal illness, reflected in high death rates (3). *S. flexneri* is a principal cause of endemic shigellosis in many developing countries, while shigellosis in both endemic and epidemic form has been attributed to *S. dysenteriae* type 1 (2). Changes in the worldwide epidemiology of *Shigella* spp. have been documented in the last decades of the 20th century. In industrialized regions, *S. dysenteriae* was first replaced by *S. flexneri*, and then by *S. sonnei* (4,5); *S. flexneri* remains the leading cause of shigellosis in most of the developing world (2,6-8).

In Indonesia, the last cases of *S. dysenteriae* diarrhea were reported in 1985 from Jakarta (8,9). This report, based on a study using a systematic surveillance approach that included a standardized detailed bacteriologic examination, provides an Indonesia-wide geographic profile of *Shigella* spp.

The Study

From June 1998 through November 1999, a total of 3,848 children and adults seeking treatment for debilitating diarrheal disease were identified from eight hospital sites in Medan, North Sumatra; Padang, West Sumatra; Batam, Riau Island; Jakarta, Java Island; Denpasar, Bali (two hospitals); Pontianak, West Kalimantan; and Makassar, South Sulawesi. Rectal swabs were obtained from patients in the study before antibiotic therapy was administered. Specimens were placed in Cary-Blair transport medium,

Address for correspondence: LCDR Buhari A. Oyofo, American Embassy Jakarta, Unit 8132 NAMRU TWO, FPO, AP 965208132, Indonesia; telephone: 62-21-421-4460; fax: 62-21-424-4507; e-mail: oyofo@namru2.org.

held at 4°C, and sent on wet ice within 2 to 4 days after collection to the U.S. Naval Medical Research Unit No. 2, Jakarta. Bacteriologic evaluation was performed by standard culture method (10). Species was confirmed by using API 20E (Biomerieux, Marcy l'Etoile, France) and slide agglutination with specific *Shigella* antisera (Difco Laboratories, Detroit, MI). Antibiotic susceptibility testing was accomplished by the disk-diffusion method (11).

Overall, bacterial isolates of S*higella* spp. were identified in 180 (5%) of 3,848 rectal swabs. The proportional contribution of *S. flexneri, S. sonnei,* and *S. dysenteriae* among shigellosis cases was 80%, 12%, and 8%, respectively. No *S. boydii* was detected. The percentage of representation among the three species did not vary substantially by geographic location. Notable was the reemergence of *S. dysenteriae* in Bali, West Kalimantan, and Batam, as well as in Jakarta after a hiatus of >15 years (Table). The proportional distribution of *S. flexneri*, *S. sonnei*, and *S. dysenteriae* for the 5- to 12-year-old and >12-year-old groups was similar. There appeared to be no consistency in the seasonal distribution patterns of *Shigella* spp. (Figure). *S. flexneri* was the most frequently isolated organism, followed by *S. sonnei* and *S. dysenteriae*.

Other enteric pathogens isolated were *Salmonella* spp. (95, 2.5%), *Vibrio cholerae* (80, 2.1%), *V. parahaemolyticus* (38, 1%), *V. cholerae* non-O1 (9, 0.2%), and *Campylobacte*r spp. (27, 0.7%). Enterotoxigenic *Escherichia coli* were detected in 225 (18.1%) of 1,240 specimens tested by the GM1 enzyme-linked immunoassay (12). Of the 541 specimens examined for rotavirus in the age groups $\leq$ 5 years, 191 (35.3%) were positive. Tests to detect parasites showed *Ascaris lumbricoides* in 8 (2%), *Blastocystis hominis* in 23 (5.6%), *Giardia lamblia* in 3 (0.7%), and *Endolimax nana* in 2 (0.5%) of 407 stool specimens examined.

Clinical presentations associated with non-*S. dysenteriae* included abdominal cramping (79%), vomiting (56%), and fever (48%); for *S. dysenteriae*, percentages of the same symptoms were 100%, 64%, and 27%, respectively. Stool samples from patients with *S. flexneri, S. sonnei, and S. dysenteriae* were principally characterized by mucus in the absence of blood (45%) or mucus and visible blood (27%).

Overall, antibiotic susceptibility patterns showed greater resistance to ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, and tetracycline for *S. flexneri* (85%, 59%, 82%, and 98%, respectively, from examination of 144 isolates) and *S. sonnei* (32%, 79%, 37%, and 100%, respectively, from examination of 22 isolates), than for *S. dysenteriae* (36%, 43%, 7%, and 29%, respectively, from examination of 14 isolates). There was no evidence of resistance to ceftriaxone, norfloxacin, ciprofloxacin, or nalidixic acid, regardless of *Shigella* species. Antimicrobial resistance was only apparent among isolates obtained from Jakarta, Bali, and Pontianak.

Conclusions

Our study showed that a substantial proportion (5%) of acute, debilitating diarrheal illness throughout Indonesia can be ascribed to shigellosis; moreover, *S. dysenteriae* was documented from various geographic locations. Both findings suggest that greater attention should be paid to highlighting the endemic and epidemic community impact of this pathogen and that laboratory detection capabilities need to be enhanced. Recognition of emerging and or reemerging disease pathogens requires reliable baseline and ongoing surveillance data.

Shifting patterns of antimicrobial-drug resistance, particularly in much of the developing world, are generally a function of overuse and

Table. Distribution of *Shigella* spp. from patients with diarrhea in Indonesia, June 1998 through November 1999

	No. specimens	No. pos. for	Proportional distribution of *Shigella* spp. (%)		
Sites	tested	*Shigella (%)*	*S. flexneri*	*S. sonnei*	*S. dysenteriae*
Jakarta	2495	122 (5)	97 (80)	17 (14)	8 (6)
Makassar	146	4 (3)	3 (75)	1 (25)	0 (0)
Denpasar (2)	607	35 (6)	29 (83)	2 (6)	4 (11)
Pontianak	330	16 (5)	13 (81)	2 (13)	1 (6)
Batam	99	1 (1)	0 (0)	0 (0)	1 (100)
Padang	51	2 (4)	2 (100)	0 (0)	0 (0)
Medan	120	0 (0)	0 (0)	0 (0)	0 (0)
Total	3848	180 (5)	144 (80)	22 (12)	14 (8)

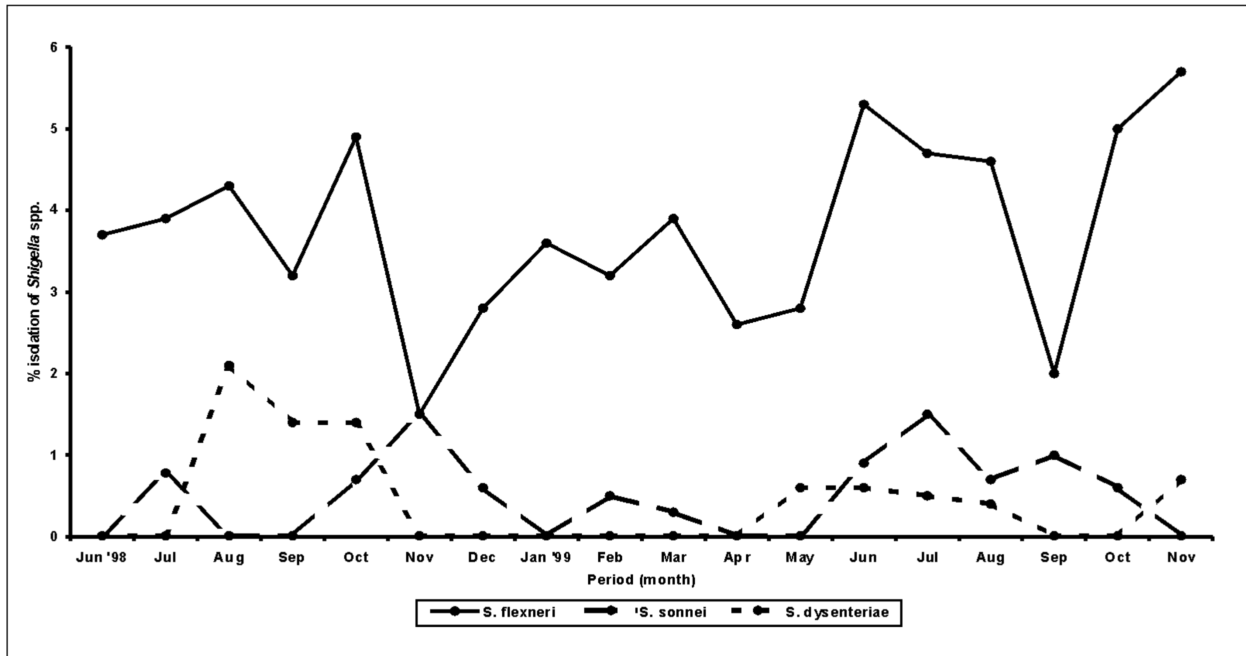


Figure. Seasonal isolates of *Shigella* spp. from patients with diarrhea in Indonesia (June 1998 - November 1999).

misuse of antibiotic drug therapies. The spread of drug resistance is the result of poorly regulated and enforced policies. Resistance to nalidixic acid (100%) among isolates of *S. dysenteriae* has been reported in Bangladesh (13); however, we found no such resistance. Nevertheless, other data from Indonesia indicate increasing resistance. In a previous report (8), 72% of *Shigella* spp. were resistant to tetracycline, but less than 30% were resistant to chloramphenicol, trimethroprim-sulfamethoxazole, or ampicillin. Previous studies from Bangladesh and Tanzania (13,6) showed that almost all tested isolates were resistant to the antibiotics used for treatment. Similar antimicrobial resistance profiles for *Shigella* spp. were reported from Thailand (14), where high resistance to ampicillin, trimethroprim-sulfamethoxazole, chloramphenicol, and tetracycline was documented.

The reemergence of *S. dysenteriae* from several locations in Indonesia should prove cause for concern to health officials, particularly in monitoring acute, debilitating diarrheal outbreaks. The epidemic potential attributed to *S. dysenteriae*, as documented in Central America, Asia, and Africa, in conjunction with notably high death rates, warrants close attention to this reemerging pathogen in Indonesia (13,15,16).

This research was supported by the U.S. Naval Medical Research and Center Command (work unit numbers 61101A-00101.EAX 2401 and 623002A810.0101.HIX.3411).

Dr. Subekti is a microbiologist and medical research scientist who heads the Molecular Microbiology Section of the Enteric Diseases Program at U.S. Naval Medical Research Unit No. 2, Jakarta, Indonesia.

References

1. Dupont HL. Shigella. Infect Dis Clin North Am 1998; 2:599-605.
2. Keusch GT, Bennish ML. Shigellosis: recent progress, persisting problems and research issues. Pediatr Infect Dis J 1989;8:713-9.
3. World Health Organization. The management of bloody diarrhea in young children 1994. Geneva: World Health Organization; 1994.
4. Ashkenazi S, May-Zahav M, Dinari G, Gabbay U, Zilberberg R, Samra Z. Recent trends in the epidemiology of *Shigella* species in Israel. Clin Infect Dis 1993;17:897-9.
5. Lee LA, Shapiro CN, Hargrett-Bean N, Tauxe RV. Hyper-endemic shigellosis in the United States: a review of surveillance data for 1967-1988. J Infect Dis 1991;64:894-900.
6. Navia MM, Capitano L, Ruiz J, Vargas M, Urassa H, Schellemberg D, et al. Typing and characterization of mechanisms of resistance of *Shigella* spp.isolated from feces of children under 5 years of age from Ifakara, Tanzania. J Clin Microbiol 1999;37:3113-7.
7. Simanjuntak CH, Hasibuan MA, Siregar LO, Koiman I. Microbial etiology of acute diarrhea (Indonesian) Health Study. Indonesia 1983;10(2):1-9.

8. Subekti D, Lesmana M, Komalarini S, Tjaniadi P, Burr D, Pazzaglia G. Enterotoxigenic *Escherichia coli* and other causes of infectious pediatric diarrheas in Jakarta, Indonesia. Southeast Asia J Trop Med Public Health 1993;24:420-4.
9. Simanjuntak CH, Hardjining S, Hasibuan MA, Pujarwoto Koiman I. Laboratory aspects of gastrointestinal infection in Indonesia, 1980-1985. Gastrointestinal Infection in Southeast SEAMIC Workshop, Japan; 1998. p. 23-31.
10. Farmer III JJ, Kelly MT. Enterobacteriaceae. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of clinical microbiology. 5th ed. Washington DC: American Society for Microbiology; 1991. p. 360-83.
11. Barry AL, Thornsberry C. Susceptibility tests: Diffusion test procedures. In: Balows A, Hausler WJ Jr, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of clinical microbiology. 5th ed. Washington DC: American Society for Microbiology; 1991. p. 1117-25.
12. Svennerholm AM, Wikstrom M, Lindbland M, Holmgren J. Monoclonal antibodies against *E. coli* heat stable toxin (Sta) and their use in a diagnostic ST ganglioside GM1-enzyme-linked immunosorbent assay. J Clin Microbiol 1986;24:585-90.
13. Sack RB, Rahman M, Yunus M, Khan EH. Antimicrobial resistance in organisms causing diarrheal disease. Clin Infect Dis 1997;24 (Suppl 1):S102-S105.
14. Moolasart P. Shigellosis in Thai people. JAMA Southeast Asia 1994;5:9-10.
15. Malakooti MA, Alaii J, Shanks GD, Phillips-Howard PA. Epidemic dysentery in western Kenya. Trans R Soc Trop Med Hyg 1997;91:541-3.
16. Mendizabal-Morris CA, Malta L, Gangarosa EJ, Guzman G. Epidemic Shiga-bacillus dysentery in Central America. Derivation of the epidemic and its progression in Guatemala, 1968-69. Am J Trop Med Hyg 1971;20:927-33.

Tracking *Cryptosporidium parvum* by Sequence Analysis of Small Double-Stranded RNA

Lihua Xiao, Josef Limor, Caryn Bern, and Altaf A. Lal
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

We sequenced a 173-nucleotide fragment of the small double-stranded viruslike RNA of *Cryptosporidium parvum* isolates from 23 calves and 38 humans. Sequence diversity was detected at 17 sites. Isolates from the same outbreak had identical double-stranded RNA sequences, suggesting that this technique may be useful for tracking *Cryptosporidium* infection sources.

Cryptosporidium parasites cause infection in humans and other vertebrates. Two genotypes of *Cryptosporidium parvum* are responsible for most cases of human infection; the human genotype (genotype 1 or anthroponotic genotype) is found almost exclusively in humans, whereas the bovine genotype (genotype 2 or zoonotic genotype) is found in both ruminants and humans (1-4). In addition to zoonotic and person-to-person transmission, both genotypes of *C. parvum* have caused waterborne and foodborne outbreaks. Current genotyping tools permit only differentiation of *Cryptosporidium* parasites at the genotype level, which limits ability to track infection and contamination sources in outbreaks.

Two double-stranded (ds) extrachromosomal viruslike RNAs have recently been identified in *C. parvum* (5). Both ds-RNAs have been found in all *C. parvum* oocysts examined. Sequence analysis of both the small and large ds-RNAs from seven *C. parvum* human genotype isolates and five bovine genotype isolates showed distinct ds-RNA sequences in isolates from the same genotype (6), indicating that ds-RNA has potential as a subgenotyping tool for *Cryptosporidium*. We report sequence diversity in the small ds-RNA of *C. parvum* human and bovine genotype isolates and discuss the usefulness of this technique for laboratory investigations and for tracking the source of cryptosporidiosis outbreaks.

Address for correspondence: Lihua Xiao, Division of Parasitic Diseases, Centers for Disease Control and Prevention, Mail Stop F12, Atlanta, GA 30333, USA; fax: 770-488-4454; e-mail: lax0@cdc.gov.

The Study

We sequenced the small ds-RNA of 61 *C. parvum* isolates (23 isolates from cattle and 38 from humans) (Table). Eighteen of the 38 human isolates were from two foodborne outbreaks (Spokane, Washington, 1997; and Washington, D.C., 1998) and one waterborne outbreak (Minnesota, 1997) with well-defined infection sources (7-9). These isolates had been genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis of the SSU rRNA and TRAP-C2 genes (10,11). All bovine isolates and the human isolates from the Minnesota outbreak were of the *C. parvum* bovine genotype, and the other human isolates were of the *C. parvum* human genotype (Table). Total nucleic acid was extracted from purified oocysts or oocyst-containing fecal materials by the phenol-chloroform method (11) and stored at -20°C before molecular analysis.

A 173-nucleotide fragment of small ds-RNA was amplified by reverse-transcription (RT)-PCR with the GeneAmp RNA PCR Core kit (PE Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Random primers were used, and the nucleic acid was preheated at 65°C for 30 min. An aliquot (2 µL) of the RT mixture was used for PCR. The primers used were 5'-TGCAGTTTACTATCCAGTGG-3' and 5'-GCAGAAGGGTTCTATGATTC-3', and the PCR conditions were those described by Khramtsov et al. (5). PCR products were sequenced on an ABI 377 Automated Sequencer (Perkin Elmer, Foster City, CA). Sequence accuracy was confirmed by two-directional sequencing and sequencing of a second RT-PCR product. Nucleotide sequences

Table. *Cryptosporidium parvum* isolates used in this study*

Isolate	Host	Source	Genotype	ds-RNA sequence type
6	Calf	Ohio, 1996[a]	Bovine	B
7	Calf	Ohio, 1996	Bovine	M
11	Calf	Ohio, 1996	Bovine	G
16	Calf	Ohio, 1996	Bovine	B
28	Calf	Ohio, 1996	Bovine	G
45	Calf	Ohio, 1996	Bovine	D
46	Calf	Ohio, 1996	Bovine	G
49	Calf	Ohio, 1996	Bovine	G
50	Calf	Ohio, 1996	Bovine	D
51	Calf	Ohio, 1996	Bovine	G
53	Calf	Ohio, 1996	Bovine	B
57	Calf	Ohio, 1996	Bovine	D
3	Calf	Oklahoma, 1996	Bovine	D
29	Calf	Oklahoma, 1996	Bovine	D
89	Calf	Pennsylvania, 1997	Bovine	D
21	Calf	Idaho, 1996	Bovine	F
37	Calf	Utah, 1996	Bovine	M
1346	Calf	California, 1999	Bovine	G
1347	Calf	California, 1999	Bovine	G
43	Human via calf	Maryland, 1996[b]	Bovine	A
Beltsville	Calf	Maryland, 1996	Bovine	H
AUCP	Calf	Alabama, 1996	Bovine	A
KSU-1	Calf	Kansas, 1996	Bovine	A
1676	Human	Peru, 1995[c]	Human	J
1677	Human	Peru, 1996	Human	J
1683	Human	Peru, 1997	Human	I
1684	Human	Peru, 1997	Human	I
1685	Human	Peru, 1997	Human	I
1902	Human	Kenya, 1999[d]	Human	Q
1904	Human	Kenya, 1999	Human	Q
1905	Human	Kenya, 1999	Human	R
1911	Human	Kenya, 1999	Human	R
1927	Human	Kenya, 1999	Human	R
1935	Human	Kenya, 1999	Human	R
HGA5	Human	Georgia, 1995	Human	N
HNO3	Human	New Orleans, 1997[e]	Human	N
HNO6	Human	New Orleans, 1997	Human	E
HNO23	Human	New Orleans, 1998	Human	P
HNO27	Human	New Orleans, 1998	Human	N
HNO30	Human	New Orleans, 1998	Human	N
HNO32	Human	New Orleans, 1998	Human	N
HNO35	Human	New Orleans, 1998	Human	O
HNO52	Human	New Orleans, 1999	Human	N
HMOB1	Human	Minnesota outbreak, 1997	Bovine	C
HMOB3	Human	Minnesota outbreak, 1997	Bovine	C
HMOB4	Human	Minnesota outbreak, 1997	Bovine	C
HMOB5	Human	Minnesota outbreak, 1997	Bovine	C
HWA1	Human	Spokane outbreak, 1997	Human	L
HWA3	Human	Spokane outbreak, 1997	Human	L
HWA4	Human	Spokane outbreak, 1997	Human	L
HWA5	Human	Spokane outbreak, 1997	Human	L
HWA6	Human	Spokane outbreak, 1997	Human	L
HDC1	Human	Washington, DC, outbreak, 1998	Human	K
HDC2	Human	Washington, DC, outbreak, 1998	Human	K
HDC6	Human	Washington, DC, outbreak, 1998	Human	K
HDC7	Human	Washington, DC, outbreak, 1998	Human	K
HDC14	Human	Washington, DC, outbreak, 1998	Human	K
HDC16	Human	Washington, DC, outbreak, 1998	Human	K
HDC23	Human	Washington, DC, outbreak, 1998	Human	K
HDC25	Human	Washington, DC, outbreak, 1998	Human	K

[a]The Ohio bovine samples were collected from four dairy farms in central Ohio over a 12-month period.
[b]The dates for laboratory isolates (Beltsville, KSU-1, and AUCP) were dates that oocyst passages were harvested for DNA extraction.
[c]Peruvian samples 1683, 1684, and 1685 were taken from the same patient on different days.
[d]Kenyan samples were collected from patients visiting two hospitals in Nairobi.
[e]The New Orleans samples were from HIV-positive patients.
ds = double-stranded

from all isolates were aligned, and the relationship between isolates was assessed by unweighted pair group method with arithmetic means, by using the Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, WI).

Eighteen distinct nucleotide sequences were obtained from the 61 isolates, dividing the 23 isolates of *C. parvum* bovine genotype into 8 subgenotypes (A,B,C,D,F,G,H, and M) and the 38 isolates of the human genotype into 10 subgenotypes (E,I,J,K,L,N,O,P,Q, and R). Subgenotype A sequence was identical to that obtained from the laboratory isolate KSU-1, whereas others showed 1- to 13-nucleotide differences from KSU-1 at 17 positions over the 173-nucleotide fragment of the small ds-RNA. Isolates of the *C. parvum* bovine genotype generally had more similarity in small ds-RNA sequences to KSU-1 (subgenotype A) than those of the *C. parvum* human genotype. However, no nucleotide changes indicative of the genotypes (bovine or human) were present in the 173-nucleotide fragment (Figure 1).

Phylogenetic analysis was inconsistent in separating isolates of the *C. parvum* bovine genotype from those of the human genotype (Figure 2). However, isolates from the same outbreak clustered together: all isolates from the Washington, D.C., outbreak (subgenotype K); the Spokane outbreak (subgenotype L); and the Minnesota outbreak (subgenotype C) had identical ds-RNA sequences (Table, Figure 2). Similarly, a subgenotype (such as subgenotypes B, N, and R) was sometimes present in several isolates from the same geographic location. Some subgenotypes (for example, D and G) had broad

```
KSU-1    TGCAGTTTACTATCCAGTGGATTTGAAATTTGTCACTGACTTATCTTCAGATCTTTCGAATACAGCTGACGGATTAGGCCAGGCTTGGTA 90
A        ..........................................................................................
B        ...............................................................G..........................
C        ...........................................................................G..............
D        ..........................................................................................
E        ..........................................................................................
F        ...............................................................G..........................
G        ...............................................................G..........................
H        ..........................................................................................
I        ...............................................................G..........................
J        ................................................G..............G..........................
K        ...............................................................G..........................
L        ..........................................................................................
M        ...............................................................G..........................
N        ...............................................................G..........................
O        ..........................................................................................
P        ................................................G......G.........................A........
Q        ................................................G......G.......G.................A........
R        ................................................G......G.........................A........

KSU-1    TAAAATTTCACAAGTCGCAGTAGAGCATATAATCCTAACGGCATTGAAGATAAATTATGTCTTGAATCATAGAACCCTTCTGC 173
A        ...................................................................................
B        ...................................................................................
C        .......................................A...........................................
D        ....................................G..A...........................................
E        ................A....C.................A...........................................
F        ...........................C...........A...........................................
G        ....................................G..A...........................................
H        ..................................T.G..A...........................................
I        .....................T.................A...........................................
J        ..................................T....A...........................................
K        ..................................T....A.................C.........................
L        C....................T............T....A...........................................
M        C.................................T.G..A.................C.........................
N        ..................................T.G..A.................C.........................
O        ..................................T.G..A.................C.........................
P        ..............CAA.G.....A..A........T..A...........................................
Q        C.............C.A.G.....A..A........C..A...........................................
R        C.............CTA.G..G..A..A........C..A...........................................
```

Figure 1. Sequence diversity in the 173-nucleotide fragment of double-stranded RNA of *Cryptosporidium parvum*. Dots denote nucleotides identical to the KSU-1 isolate of the *C. parvum* bovine genotype. Representative sequences for each subgenotype were deposited in GenBank under accession numbers AF266262 to AF266277.

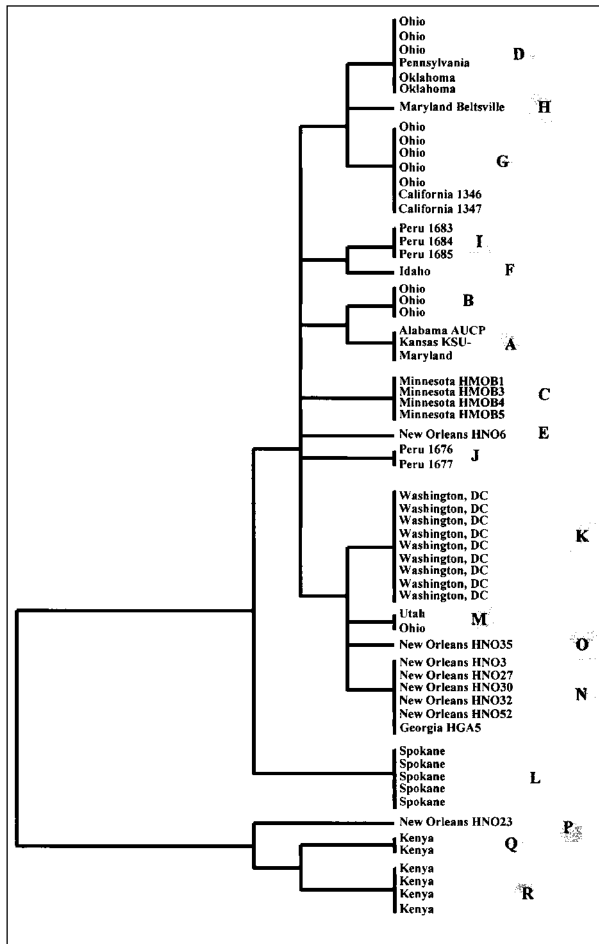


Figure 2. Genetic relationships of various subgenotypes of *Cryptosporidium parvum* human and bovine genotypes inferred by the unweighted pair group method with arithmetic means analysis of the small double-stranded RNA.

geographic distribution, and isolates from a given geographic area (such as those from calves in Ohio and humans in New Orleans) frequently had several subgenotypes.

Conclusions

Subgenotyping tools are needed for studies of the molecular epidemiology of cryptosporidiosis. Such tools would facilitate laboratory characterization of cryptosporidiosis outbreaks and identification of contamination and infection sources. Analysis of the variations in subgenotype occurrence may also shed light on the transmission dynamics of *Cryptosporidium* parasites in different geographic areas and epidemiologic settings. The extensive intragenotypic heterogeneity in the small ds-RNA sequence exhibited by isolates of the *C. parvum* bovine and human genotypes indicates that ds-RNA has potential as a high-resolution tool for subgenotyping *Cryptosporidium* parasites.

Our analysis of outbreak specimens illustrates the potential utility of subgenotyping tools for epidemiologic investigations. The waterborne outbreak in Minnesota affected children who played around a water fountain in a zoo (7). All four isolates had the same subgenotype (C), confirming that the children's infections came from the same source. In the foodborne outbreak in Spokane, which affected attendees at a holiday party (8), all five isolates analyzed had the subgenotype L sequence of the *C. parvum* human genotype, supporting the epidemiologic conclusion of a single source. The outbreak in Washington, D.C., was attributed to contamination of food by a food-handler who had symptomatic cryptosporidiosis in the week before the outbreak (9). As in the other outbreaks, all eight isolates were of the subgenotype K of the *C. parvum* human genotype, again confirming a common source. Analysis of a sample (HDC14) from the food-handler demonstrated a small ds-RNA sequence identical to those from the outbreak cases, providing further evidence that the food-handler was the likely source of the oocysts that caused the outbreak.

The presence of multiple subgenotypes at the same geographic location, the wide distribution of certain subgenotypes, and the apparent geographic segregation of some subgenotypes seen in this preliminary study highlight the complexity of cryptosporidiosis epidemiology. The two subgenotypes of *C. parvum* in Kenya were quite divergent from isolates from other areas, which suggests localized transmission cycles. This hypothesis is further supported by the predominance of one subgenotype (N) in New Orleans AIDS patients. However, the presence of four subgenotypes (B,D,G, and M) of the *C. parvum* bovine genotype in calves in central Ohio suggests that multiple *C. parvum* parasites of the same genotype can circulate simultaneously in a region. Both phenomena may occur in any given locality, leading to the pattern seen in eight specimens from AIDS patients in New Orleans, where five specimens were subgenotype N and the other three specimens were of three different subgenotypes. Analysis of more isolates from diverse locations is needed for a firm extrapolation of data.

A disadvantage of the ds-RNA subgenotyping tool is lack of specificity at the genotype level. Perhaps as a result of the use of a short fragment as the target, this technique does not distinguish the two genotypes of *C. parvum* and must therefore be used in combination with routine genotyping tools. Initial attempts targeted longer fragments of the large and small ds-RNAs. However, the RT-PCR that targeted longer fragments in amplifying samples of the *C. parvum* human genotype was much less efficient, probably because of sequence diversity at the primer regions and lower efficiency of reverse transcription of longer fragments. A recent sequence analysis by Khramtsov et al. of five isolates of the *C. parvum* bovine genotype and seven isolates of the human genotype consistently separated the two genotypes in both the large and small ds-RNAs (6). It remains to be determined whether these primers and others can be developed for sensitive genotyping and subgenotyping *Cryptosporidium* parasites.

Acknowledgments

We thank Anne Moore, Barbara Herwaldt, Michael Arrowood, Bruce Anderson, William Shulaw, A. Morse, J. Inungu, Ronald Fayer, Robert H. Gilman, Lilia Cabrera, William Checkley, and Wangeci Ndiritu for providing *Cryptosporidium* isolates.

This work was supported in part by funds from the Food Safety Initiative, Centers for Disease Control and Prevention.

Dr. Xiao is a senior staff fellow in the Division of Parasitic Diseases, CDC. His research interests focus on the molecular epidemiology of enteric protozoa and malaria vaccine development.

References

1. Morgan UM, Constantine CC, O'Donoghue P, Meloni BP, O'Brien PA, Thompson RCA. Molecular characterization of *Cryptosporidium* isolates from humans and other animals using random amplified ploymorphic DNA analysis. Am J Trop Med Hyg 1995;52:559-64.
2. Bonnin A, Fourmaux MN, Dubremetz JF, Nelson RG, Gobet P, Harly G, et al. Genotyping human and bovine isolates of *Cryptosporidium parvum* by polymerase chain reaction-restriction fragment length polymorphism analysis of a repetitive DNA sequence. FEMS Microbiol Lett 1996;137:207-11.
3. Peng MP, Xiao L, Freeman AR, Arrowood MJ, Escalante A, Weltman AC, et al. Genetic polymorphism among *Cryptosporidium parvum* isolates supporting two distinct transmission cycle. Emerg Infect Dis 1997;3:1-9.
4. McLauchlin J, Pedraza-Diaz S, Amar-Hoetzeneder C, Nichols GL. Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. J Clin Microbiol 1999;37:3153-8.
5. Khramtsov NV, Woods KM, Nesterenko MV, Dykstra CC, Upton SJ. Virus-like, double-stranded RNAs in the parasitic protozoan *Cryptosporidium* parvum. Mol Microbiol 1997;26:289-300.
6. Khramtsov NV, Chung PA, Dykstra CC, Griffiths JK, Morgan UM, Arrowood MJ, et al. Presence of double-stranded RNAs in human and calf isolates of *Cryptosporidium* parvum. J Parasitol 2000;86:275-82.
7. Centers for Disease Control and Prevention. Outbreak of cryptosporidiosis associated with a water sprinkler fountain–Minnesota, 1997. MMWR Morb Mortal Wkly Rep 1998;47:856-9.
8. Centers for Disease Control and Prevention. Foodborne outbreak of cryptosporidiosis–Spokane, Washington, 1997. MMWR Morb Mortal Wkly Rep 1998;47:565-6.
9. Quiroz ES, Bern C, MacArthur JR, Xiao L, Fletcher M, Arrowood MJ, et al. An outbreak of cryptosporidiosis linked to a foodhandler. J Infect Dis 2000;181:695-700.
10. Sulaiman IM, Xiao L, Yang C, Moore A, Beard CB, Arrowood MJ, et al. Differentiating human from animal isolates of *Cryptosporidium parvum*. Emerg Infect Dis 1998;4:681-5.
11. Xiao L, Morgan U, Limor J, Escalante A, Arrowood M, Shulaw W, et al. Genetic diversity within *Cryptosporidium parvum* and related species of *Cryptosporidium*. Appl Environ Microbiol 1999;65:3386-91.

Pathologic Studies of Fatal Cases in Outbreak of Hand, Foot, and Mouth Disease, Taiwan

Wun-Ju Shieh,* Shih-Ming Jung,† Chuen Hsueh,† Tseng-Tong Kuo,† Anthony Mounts,* Umesh Parashar,* Chen Fu Yang,* Jeannette Guarner,* Thomas G. Ksiazek,* Jacqueline Dawson,* Cynthia Goldsmith,* Gwong-Jen J. Chang,* Steve M. Oberste,* Mark A. Pallansch,* Larry J. Anderson,* Sherif R. Zaki,* and the Epidemic Working Group[1]

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Chang Gung Memorial Hospital, Tauyuan, Taiwan, Republic of China

In 1998, an outbreak of enterovirus 71-associated hand, foot, and mouth disease occurred in Taiwan. Pathologic studies of two fatal cases with similar clinical features revealed two different causative agents, emphasizing the need for postmortem examinations and modern pathologic techniques in an outbreak investigation.

During April through July 1998, an outbreak of hand, foot, and mouth disease occurred in Taiwan; enterovirus 71 (EV71) was identified as the main etiologic agent. The outbreak was associated with an unusually high death rate in young children. At least 55 fatal cases were initially reported (1,2) in healthy children, who had refractory shock after an acute prodromal illness; many of them manifested neurologic disorders during illness and died within 24 hours of hospitalization (3). In many fatal cases, EV71 was indicated as the etiologic agent by serologic, virologic, and polymerase chain reaction tests conducted on specimens from nonsterile sites, such as throat swabs or stool specimens. While these results may indicate a precedent EV71 infection in the fatal cases, they do not directly implicate EV71 as the causative agent of neurologic disorders and eventual death. In contrast, histopathologic examination in conjunction with special pathology techniques, such as immunohistochemistry (IHC), in situ hybridization, and electron microscopy, can provide unequivocal evidence linking a particular agent to death. We report results of pathologic examination of two fatal cases during this outbreak.

Address for correspondence: Wun-Ju Shieh, Centers for Disease Control and Prevention, 1600 Clifton Road, Mail Stop G32, Atlanta, GA 30333, USA: fax: 404-639-3043; e-mail: wshieh@cdc.gov.

The Study

The clinical and histopathologic features of the first case were initially reported by Chang et al. (4); the second case was in a patient admitted to the same hospital with a similar clinical course. Histopathologic features were similar in both cases and showed severe and extensive encephalomyelitis. An IHC technique using a monoclonal mouse anti-EV71 antibody and an in situ hybridization test using a digoxigenin-labeled enterovirus probe were performed on formalin–fixed, paraffin-embedded central nervous system (CNS) tissues and major organs of both patients. In the first case, positive staining of enteroviral antigens and nucleic acids was observed in neurons, neuronal processes, and inflammatory foci at various CNS sites, including the cerebral cortex, brain stem, and all levels of spinal cord (Figure 1A, B). No immunostaining or hybridization was present in lung, heart, liver, spleen, or kidney. Electron microscopy evaluation of spinal cord tissues showed a highly vacuolated neuron containing scattered picornavirus-like particles and viral inclusions (Figure 1C).

[1]Epidemic Working Group at Centers for Disease Control and Prevention: Jim Alexander, Betty Brown, David Shay, Patricia Greer, Charles Humphrey, Tim Morken, and Tara Ferebee-Harris.

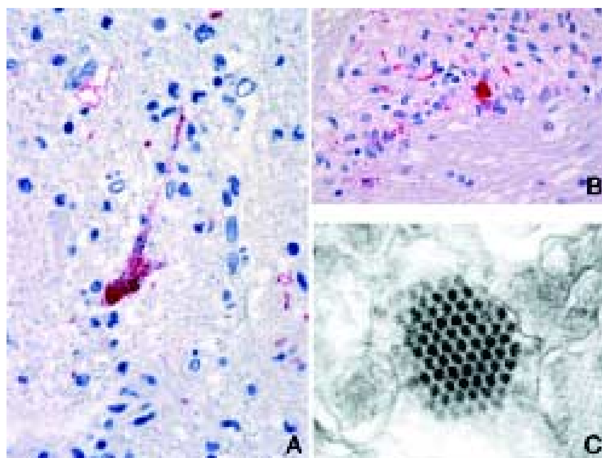


Figure 1. A). Positive immunostaining of EV71 antigens in neuron and neuronal process. Original magnification, X158. B). Positive immunostaining of EV71 antigens in necrotic area. Original magnification, X158. C). An array of picornavirus particles in a neuron (electron micrograph).

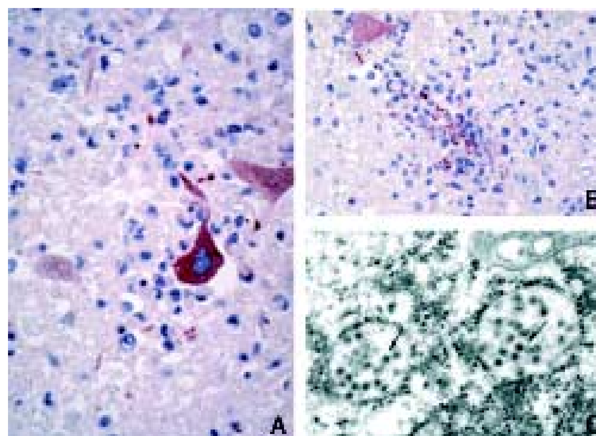


Figure 2. A). Positive immunostaining of Japanese encephalitis antigens in neuron and neuronal process. Original magnification, X158. B). Positive immunostaining of Japanese encephalitis antigens in necrotic area. Original magnification, X158. C). Flavivirus particles (arrowheads) in isolates from mouse brain (electron micrograph).

The second case was negative for EV71 by IHC, in situ hybridization, polymerase chain reaction, and viral isolation. An IHC test using an anti-Japanese encephalitis antibody showed intense immunostaining of flaviviral antigens in neurons, neuronal processes, and inflammatory foci at various CNS sites (Figure 2A, B). No immunostaining of flavivirus was present in other major organs. Further study was conducted by injecting neurologic tissue of the patient into suckling mice. CNS material from an inoculated mouse was passed onto Vero-E6 cells and produced cytopathic effect. Electron microscopy examination of mouse brain and infected cell culture revealed flavivirus particles (Figure 2C), and polymerase chain reaction with sequencing confirmed the isolate as Japanese encephalitis virus.

Conclusions

In addition to classic hand, foot, and mouth disease, EV71 can cause severe CNS infections with a high death rate (5). Two previous EV71 outbreaks were associated with neurologic disorders and increased deaths; the first one occurred in Bulgaria in 1975 (5) and the second in Malaysia in 1997 (1,6). The neurologic disorders and clinical courses of acute viral CNS infections can be very similar regardless of causative agents, and definitive diagnoses are sometimes very difficult to establish without further pathologic studies. A definitive laboratory and pathologic diagnosis is crucial for implementing effective measures to control an outbreak (7); the Nipah virus encephalitis outbreak in Malaysia (8,9) and the West Nile encephalitis outbreak in New York City (10,11) lend additional support to this statement.

Our report illustrates how two different etiologic agents can cause similar diseases during an outbreak and emphasizes the need for postmortem examination.

Dr. Shieh is a staff pathologist, Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention. His research interests include infectious disease pathology, pathology and pathogenesis of viral encephalitides, molecular epidemiology, and infectious disease outbreak investigations.

References

1. Centers for Disease Control and Prevention. Deaths among children during an outbreak of hand, foot, and mouth disease –Taiwan, Republic of China, April-July 1998. MMWR Morb Mortal Wkly Rep 1998;47:629-32.
2. Ho M, Chen ER, Hsu KH, Twu SJ, Chen KT, Tsai SF, et al. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N Engl J Med 1999;341:929-35.
3. Liu C, Tseng H, Wang S, Wang J, Su I. An outbreak of enterovirus 71 infection in Taiwan, 1998: epidemiologic and clinical manifestations. J Clin Virol 2000;17:23-30.
4. Chang LY, Huang YC, Lin TL. Fulminant neurogenic pulmonary oedema with hand, foot, and mouth disease. Lancet 1998;352: 367-8.

5. Shindarov L, Chumakov M, Voroshilova M, Bojinov S, Vasilenko SM, Iordanov I, et al. Epidemiological, clinical and pathomorphological characteristics of epidemic poliomyelitis-like disease caused by enterovirus 71. J Hyg Epidemiol Microbiol Immunol 1979; 23: 284-95.
6. Lum LCS, Wong KT, Lam SK, Chua KB, Goh AY, Lim WL, et al. Fatal enterovirus 71 encephalomyelitis. J Pediatr 1998;133:795-8.
7. Zaki SR, Shieh W-J, and the Epidemic Working Group at Ministry of Health in Nicaragua, et al. Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995. Lancet 1996;347:535-6.
8. Centers for Disease Control and Prevention. Outbreak of Hendra-like virus—Malaysia and Singapore, 1998–1999. MMWR Morb Mortal Wkly Rep 1999;48:265–9.
9. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, et al. Nipah virus: a recently emergent deadly Paramyxovirus. Science 2000;288:1432-5.
10. Centers for Disease Control and Prevention. Outbreak of West Nile-like viral encephalitis--New York, 1999. MMWR Morb Mortal Wkly Rep 1999;48:845-9.
11. Shieh WJ, Guarner J, Layton M, Fine A, Miller J, Nash D, et al. The role of pathology in an investigation of an outbreak of West Nile encephalitis in New York, 1999. Emerg Infect Dis 2000;6:370-2.

Disseminated *Neocosmospora vasinfecta* Infection in a Patient with Acute Nonlymphocytic Leukemia

Oliver A. Cornely,* Jens Chemnitz,* Hans-Georg Brochhagen, Karin Lemmer,† Heidi Schütt, Dietmar Söhngen,* Peter Staib,* Claudia Wickenhauser,* Volker Diehl,* and Kathrin Tintelnot†
*Universitätsklinik Köln, Köln, Germany, and
†Robert Koch-Institute, Berlin, Germany

We report *Neocosmospora vasinfecta* infection following chemotherapy for acute nonlymphocytic leukemia. *N. vasinfecta*, a plant pathogen, was identified by culture and genetic sequencing. Susceptibility testing revealed in vitro resistance for common antifungals.

Neocosmospora vasinfecta, a common plant pathogen in tropical and subtropical areas (1), is little known as a pathogen in humans. We report a case of fatal systemic *N. vasinfecta* infection in an African patient with leukemia. This is the third reported case of a human infection with this organism and the first case in which the infection was disseminated.

The Case Study

A 38-year-old man was admitted to the University Hospital of Cologne, Germany, with a 4-week history of fatigue, vertigo, and bone pain. He had been living in Germany for 15 years and had returned from a holiday in Nigeria 2 weeks before admission. Past medical history included hepatitis C and malaria.

A bone marrow aspirate showed a predominance of atypical promyelocytes, suggesting acute nonlymphocytic leukemia FAB M3. The patient was treated with high-dose all-trans-retinoic acid for 14 days, followed by a first induction chemotherapy regimen consisting of cytarabine, daunorubicine, and thioguanine. Prophylactic treatment with fluconazole (400 mg/d) and trimethoprim-sulfamethoxazole (960 mg/d) was started because severe granulocytopenia, defined as an absolute neutrophil count below 100/µL of up to 4 weeks' duration, was anticipated.

Address for correspondence: Oliver A. Cornely, Klinik I für Innere Medizin, Universitätsklinik Köln, 50924 Köln, Germany; fax:+49-221-478-3611; e-mail: oliver.cornely@uni-koeln.de.

Pain projecting to the lateral side of both feet developed on day 12 of chemotherapy, when the patient's absolute neutrophil count had fallen below 100/µL. His feet were swollen and hyperthermic. Arthritis and deep vein thrombosis were ruled out. Pain resolved under symptomatic treatment with acetaminophen. On day 16 of induction therapy, bone marrow examination yielded persistence of blast cells. Therefore, a second induction chemotherapy was administered, despite ongoing granulocytopenia. The regimen comprised high-dose cytarabine and mitoxantrone. On day 16 after the onset of granulocytopenia, fever developed. Results of a chest X-ray and a high-resolution computerized tomography scan were unremarkable. Broad-spectrum antibiotic treatment was started, but the fever persisted. Several chest X-rays were nondiagnostic; however, after 24 days of granulocytopenia, another computerized tomography scan revealed interstitial and patchy lung infiltrates. Because of persistent fever and newly developing lung infiltrates under broad-spectrum antibiotic therapy, invasive fungal infection was suspected, and conventional amphotericin B was administered (1 mg/kg/d). After 1 week of antifungal therapy, the patient reported bilateral pleuritic chest pain. Because the patient's condition was deteriorating, antifungal therapy was altered to 2 mg/kg/day liposomal amphotericin B, but pulmonary infiltrates persisted. Granulocytopenia resolved after 34 days, and a bone marrow aspirate showed complete remission of

leukemia. On the same day, an abdominal ultrasound exam revealed hepatosplenomegaly and hypodensic lesions in the liver, suggesting possible hepatosplenic involvement of fungal disease. A lateral purulent ulcerous lesion of the right foot developed, accompanied by worsening pain and swelling. Swab cultures revealed a mold that was not initially identified. Subsequently, the same mold was isolated from sputum samples, transtracheal aspirates, a second biopsy of the foot lesion (Figure 1), and blood cultures. The patient's condition deteriorated rapidly; cholestasis and septic shock developed, and mechanical ventilation and high doses of vasopressors were required. Fifteen days after resolution of neutropenia and while undergoing antimycotic treatment, the patient died of multiorgan failure caused by systemic fungal infection. Consent was not given for autopsy.

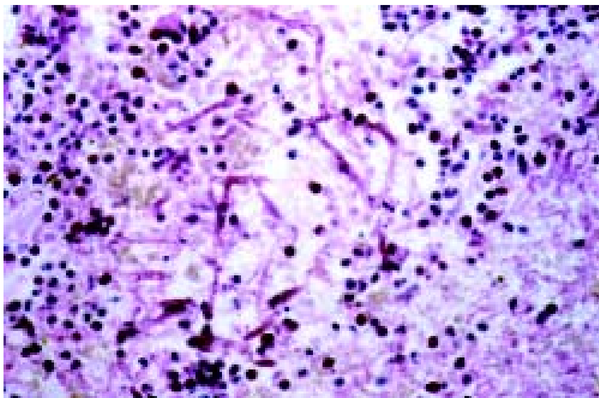


Figure 1. Biopsy of foot lesion: area of extended dermal necrosis with inflammatory infiltration. In the center, PAS-(periodic acid-Schiff stain)positive septate hyphae branching at an acute angle (x400).

Samples were cultivated on Sabouraud agar at 30°C and 37°C in ambient air. Subcultures were prepared on malt yeast agar, oatmeal agar, and potato dextrose agar. The fast-growing, white-to-pale-buff colonies developed at 26°C and 37°C without special requirements.

The micromorphologic features of the mold, cultured 3 to 4 days only, resembled a *Fusarium* or *Acremonium* species (Figure 2a). Supported by subculturing on oatmeal agar with *Lupinus* sp., orange-brown to copper-colored fruiting bodies developed until day 8 (Figure 2b); after 14 days of incubation, these bodies were identified as perithecial ascomata (diameter 200 µm-300 µm). They contained hyaline cylindrical asci, 9 x 80-90 µm, with 8 ascospores each (Figure 2c); the latter were globose to ellipsoidal, pale brown, rough walled with age, and 8-9 x 10 µm. The isolate was subsequently identified at the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, as a small-spored *N. vasinfecta* E.F. Smith var. *vasinfecta* and was deposited under the collection number CBS 101957.

Sequence analysis of the internal transcribed spacer (ITS) region inside the nuclear rDNA was performed. DNA was extracted by sonication in CTAB buffer (cetyl trimethyl ammonium bromide) (2) and amplified with the primer pair ITS5/ITS4. The polymerase chain reaction

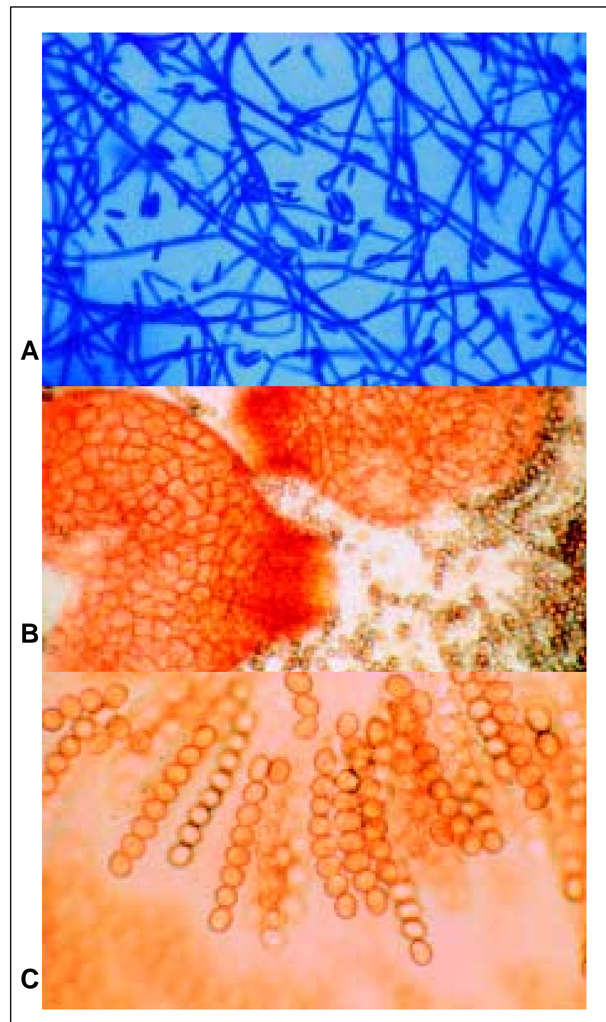


Figure 2. A) *Neocosmospora vasinfecta* after 4 days' subculture on Sabouraud agar (lactophenol cotton blue stain, x400). B) *N. vasinfecta* perithecial ascomata after 4 weeks on oatmeal agar with lupine stem (x160). C) Asci with ascospores of *N. vasinfecta* (x400).

product was purified from agarose gel by using the Gel Extraction Kit (Qiagen GmbH, Hilden, Germany) and directly sequenced with the primers ITS2, ITS3, ITS4, and ITS5 (3). DNA sequencing was performed by the dideoxynucleotide termination method, using the dye deoxy terminator chemistry and the ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA). The results were compared with the published ITS sequence, GenBank accession no. L36627.

When we compared our results to the published 544-bp ITS sequence, we found differences in three positions: T to C exchange at nucleotide position 72, C to T exchange at position 102, and an insertion of a seventh C between positions 381 and 388.

The MICs of amphotericin B and 5-flucytosine were determined by the ATB Fungus kit (Biomérieux SA, Marcy l'Étoile, France). Susceptibility testing to fluconazole, itraconazole, terbinafine, and voriconazole (Pfizer Inc., New York, NY) was performed by using a casitone-based microdilution system (4). The MIC for itraconazole was simultaneously determined according to the National Committee for Clinical Laboratory Standards (NCCLS) M27-A protocol (5). The inoculum had a final amount of $1x10^4$ conidia per microliter. Incubation time at 35°C was 44 hours for amphotericin B and 5-flucytosine and 30 hours for the other antifungals.

MICs were as follows: amphotericin B > 8 µg/mL^{-1}, 5-flucytosine >128 µg/mL^{-1}, fluconazole >128 µg/mL^{-1}, itraconazole >2 µg/mL^{-1} versus >8 µg/mL^{-1} (according to NCCLS), voriconazole 1 µg/mL^{-1} , and terbinafine 0.125 µg/mL^{-1}.

Conclusions

In the early days of incubation, the micromorphologic features of *N. vasinfecta* isolates may be misleading, suggesting *Fusarium* or *Acremonium* species (1), which could lead to an inadequate therapeutic regimen because of the different amphotericin B susceptibility of these genera. While high-dose amphotericin B intravenously is the first-line therapy in systemic fusarioses, polyene antimycotics are probably not beneficial against infections caused by *N. vasinfecta*, as demonstrated in our patient and in a recently reported immunocompetent patient with posttraumatic *N. vasinfecta* infection (6).

Our patient died of disseminated infection by *N. vasinfecta* despite high-dose antimycotic therapy with amphotericin B and remission of the underlying hematologic malignancy. The in vitro susceptibility test results confirmed the resistance of the isolate against amphotericin B and fluconazole and nearly all of the few other antifungal agents available for systemic therapy. Not enough data on terbinafine are available to allow valid interpretation. In comparison to the MICs of voriconazole against other filamentous fungi such as *Aspergillus fumigatus* (7), the susceptibility for voriconazole in our isolate might not have been sufficient for adequate antifungal treatment.

Neither the mechanism of infection nor the incubation period could be determined. Our patient did not remember any injury, so infection most likely occurred through minimal trauma. Although he might have been infected during his recent stay in Nigeria, infection might have occurred in Africa considerably longer ago: The latency period for *N. vasinfecta* can be several years between infection and the symptomatic stage (8). The infection might never have been detected had the patient remained immunocompetent. Once the infection became systemic, it was likely incurable.

The two previously reported patients (6,8,9), both from Senegal, had been cured by a radical surgical intervention that prevented dissemination of disease, limiting it to the lower extremities. Because of the poor susceptibility of the fungus, complete surgical excision prompted by early diagnosis is the only means to influence the clinical outcome. Currently, no medical treatment option exists for a patient with systemic disease caused by *N. vasinfecta*.

Imported mycoses should be taken into consideration in patients who have traveled or lived abroad, especially former residents of tropical and subtropical countries, even years after their stay abroad. Recent epidemiologic data on plants suggest that persons working in cotton production might also be at risk (10).

This is the first case of disseminated *N. vasinfecta* infection. Physicians may consider this infection as a new differential diagnosis of purulent subcutaneous leg lesions in the severely immunocompromised host.

Acknowledgments

The authors thank Michael Seibold for performing the antifungal susceptibility tests.

Dr. Cornely is a senior resident and clinical research fellow at the Universitätsklinik Köln, Köln, Germany. His research interests include treatment of hematologic and oncologic malignancies, with a focus on supportive care and infectious diseases in neutropenic hosts.

References

1. Cannon PF, Hawksworth DL. A revision of the genus Neocosmospora (Hypocreales). Transactions of the British Mycological Society 1984;82:673-88.
2. Wedde M, Müller D, Tintelnot K, De Hoog GS, Stahl U. PCR-based identification of clinically relevant Pseudallescheria/Scedosporium strains. Med Mycol 1998; 36:61-7.
3. White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis N, Gelfand J, White T, editors. PCR protocols: a guide to methods and applications. New York: Academic Press; 1990. p. 315-22.
4. Seibold M, Werner E. Testing susceptibility of Candida species to fluconazole and itraconazole using the microdilution assay. Mycoses 1995;38:443-8.
5. National Committee for Clinical Laboratory Standards (NCCLS). Reference method for broth dilution antifungal susceptibility testing of yeasts. M27-A. Villanova, PA: NCCLS; 1997.
6. Kac G, Piriou P, Gueho E, Roux P, Tremoulet J, Denis M, et al. Osteoarthritis caused by *Neocosmospora vasinfecta*. Med Mycol 1999;37:213-7.
7. Espinel-Ingroff A. In vitro activity of the new triazole voriconazole (UK-109,496) against opportunistic filamentous and dimorphic fungi and common and emerging yeast pathogens. J Clin Microbiol 1998;36:198-202.
8. Ben Hamida F, Achard JM, Westeel PF, Chandenier J, Bouzernidj M, Petit J, et al. Leg granuloma due to *Neocosmospora vasinfecta* in a renal graft recipient. Transplant Proc 1993;25:2292.
9. Chandenier J, Hayette MP, de Bièvre C, Westeel PF, Petit J, Achard JM, et al. Tuméfaction de la jambe à Neocosmospora vasinfecta chez un transplanté rénal. Journal de Mycologie Médicale 1993;3:165-8.
10. Baird R, Carling D. Survival of parasitic and saprophytic fungi on intact senescent cotton roots. Journal of Cotton Science 1998;2:27-34.

Adventitious Viral Genomes in Vaccines but Not in Vaccinees

It is a pleasant change to write about viruses that might have emerged but haven't. In this issue, Hussain and colleagues at the Centers for Disease Control and Prevention, the U.S. Department of Agriculture, and Harvard University report that recipients of measles, mumps, and rubella (MMR) vaccine show no evidence of infection by endogenous avian retroviruses, even though viral genomes and reverse transcriptase activity have been detected in vaccine preparations. Influenza, yellow fever, and MMR vaccines are usually prepared in embryonated eggs or in cultures of chick embryo fibroblasts (CEF). These fibroblasts contain and express endogenous retroviral genomes (1). In any vaccine, adventitious agents in the cellular substrate may contaminate the biological product. In live, attenuated vaccines, such contaminants are not inactivated, and endogenous retroviruses by their very nature as Mendelian transmitted genomes are particularly difficult to eliminate. Endogenous retrovirus release also has ramifications for pharmaceutical proteins made in cell substrates (e.g., monoclonal antibodies) and for xenotransplantation (2,3).

Some 45 years ago, it was found that apparently healthy hens could transmit avian leukosis virus (ALV) vertically in eggs (4); later it was demonstrated that live virus vaccines made in CEF were contaminated with infectious ALV (5). However, no increased risk for cancer was found in yellow fever vaccinees with the longest presumed exposure to ALV (6). Nevertheless, vaccine manufacturers were soon required to use eggs or CEF from leukosis-free flocks. To screen for ALV infection, a complement fixation for ALV (COFAL) antigen test was devised, and through pioneering work in the 1960s, the existence of endogenous retroviruses came to light because many ALV-free birds were COFAL positive (7-9).

As a graduate student at the time, I observed that CEF of COFAL-positive embryos complemented envelope-defective Rous sarcoma virus, yielding pseudotype viruses with xenotropic properties. The endogenous virus was genetically transmitted in chickens but was infectious for other hosts such as quail and pheasant. Many copies of partial or complete ALV genomes were located in chicken DNA (1). We showed that ALV had colonized the host germ line of red jungle fowl before domestication to become chickens but after divergence of the genus *Gallus* into distinct species. Even so, it proved possible in the 1970s to breed white leghorns free of endogenous ALV genomes; such chickens are now being introduced by Merck as preferred substrates for vaccine production.

A second class of endogenous avian retroviral genome (EAV), discovered in 1985 (10), is present in all breeds of chicken and cannot be eliminated. EAV can release noninfectious virus particles containing active reverse transcriptase; and this is the genome most commonly found in MMR and other vaccines (Hussain et al., this issue; 11). The major retroviral pathogen of meat-strain chickens is an infectious recombinant between ALV *gag* and *pol* genes and an *env* gene related to EAV (12). This virus has not been observed to infect human cells.

May we assume, therefore, that chicken cell substrate vaccines are safe? With biological products, as with crossing the street, there is no such thing as absolute safety. The paper by Hussain et al. is reassuring, and I agree with the authors that no change in current U.S. policies (or WHO policies, for that matter) is warranted, and the public should continue to enjoy the benefit of the vaccine. However, it may be useful to probe the possibility of interaction between endogenous avian viruses and the infectious components of MMR. We showed that vesicular stomatitis virus (VSV) could assemble its glycoprotein G on avian retrovirus virions and vice versa (13). Indeed, VSV G protein has become an envelope of choice for retroviral vectors developed for gene therapy. By analogy, the assembly of the hemagglutinin and fusion glycoproteins of measles or mumps viruses might confer a human host range on endogenous ALV or EAV particles. The possible generation of such pseudotypes or phenotypically mixed virions in vaccines may be worthy of investigation.

In addition, with ultrasensitive techniques, such as polymerase chain reaction (PCR) gene amplification, we can detect viral genomes and reverse transcriptase activity more readily in vaccine preparations. Virtually all vertebrates studied, including humans, carry endogenous retroviral genomes as part of their natural

genetic constitution (1,14). Therefore, almost any cell substrate for vaccine production (avian, rodent, or primate) is likely to contain and express (at low level) endogenous retroviral genomes.

Vaccine contamination by adventitious viruses in the cellular substrate has, of course, occurred before. In one instance, the discovery of SV40 in rhesus macaque kidney cultures (15) soon led to the adoption of cynomolgus macaque and later African green monkey (AGM) kidneys as the preferred substrate for polio vaccines. That was, perhaps, a near escape as AGMs are now known to frequently harbor a strain of simian immunodeficiency virus (SIV) that luckily does not appear to infect humans. Following the potential exposure of millions of polio vaccinees to SV40, no evidence was found of increased cancer incidence (16). More recently, it has been reported that SV40 is present in some human cancers (17). Cases include pediatric tumors in patients born long after SV40 was eliminated from polio vaccines.

Ironically, it was the misguided attention of regulatory groups on hypothetical oncogenic DNA that led to vaccine contamination by adventitious oncogenic viruses in the first place. Fear of oncogenic DNA made tumor cell lines taboo as cellular substrates for vaccine production. Despite all we have learned about oncogenes and tumor suppressor genes in multistep progression to cancer, the possible trace of "oncogenic" DNA in vaccines prepared in established cell lines remained of greater concern to regulators than adventitious infections in primary cells. It is high time to reevaluate the relative risks, so it is heartening that the Food and Drug Administration held a workshop last year to begin that process.

Robin A. Weiss
University College London, London, UK

References

1. Coffin J. Endogenous viruses. In: Weiss RA, Teich NM, Varmus HE, Coffin J, editors. RNA tumor viruses. New York: Cold Spring Harbor Laboratory Press;1982.p. 1109-203.
2. Weiss RA. Retroviruses produced by hybridomas. N Engl J Med 1982;307:1587.
3. Patience C, Takeuchi Y, Weiss RA. Infection of human cells by an endogenous retrovirus of pigs. Nat Med 1997;3:282-6.
4. Burmester BR, Gentry RF, Waters NF. The presence of the virus of visceral lymphomatosis in embryonated eggs of normal appearing hens. Poultry Sci 1955;34:609-17.
5. Dougherty RM, Harris RJ, Biggs PM, Payne LN, Goffe AP, Churchill AE, et al. Contaminant viruses in two live virus vaccines produced in chick cells. J Hyg 1966;64:1-7.
6. Waters TD, Anderson PS, Beebe GW, Miller RW. Yellow fever vaccination, avian leukosis virus, and cancer risk in man. Science 1972;177:76-7.
7. Dougherty RM, DiStefano HS. Lack of relationship between infection with avian leukosis virus and the presence of COFAL antigen in chick embryos. Virology 1966;29:586-95.
8. Dougherty RM, DiStefano HS, Roth FK. Virus particles and viral antigens in chicken tissues free of infectious avian leukosis virus. Proc Natl Acad Sci U S A 1967;58:808-17.
9. Payne LN, Chubb RC. Studies on the nature and genetic control of an antigen in normal chick embryos which reacts in the COFAL test. J Gen Virol 1968;3:379-91.
10. Dunwiddie C, Faras AJ. Presence of retrovirus reverse transcriptase-related gene sequences in avian cells lacking endogenous avian leukosis viruses. Proc Natl Acad Sci U S A 1985;82:5097-101.
11. World Health Organization. Reverse transcriptase activity in chicken-cell derived vaccine. Wkly Epidemiol Rec 1998;73:209-12.
12. Bai J, Payne LN, Skinner MA. HPRS-103 (exogenous avian leukosis virus, subgroup J) has an env gene related to those of endogenous elements EAV-0 and E51 and an E element found previously only in sarcoma viruses. J Virol 1995;69:779-84.
13. Weiss RA, Boettinger DE, Love D. Phenotypic mixing between vesicular stomatitis virus and avian RNA tumor viruses. Cold Spring Harbor Symp Quant Biol 1975;39:913-8.
14. Patience C, Wilkinson DA, Weiss RA. Our retroviral heritage. Trends Genet 1997;13:116-20.
15. Sweet BH, Hilleman MR. The vacuolating virus, SV40. Proc Soc Exp Biol Med 1960;105:420-7.
16. Nathanson N, Shah K. Human exposure to SV40: review and comment. Am J Epidemiol 1976;103:1-12.
17. Butel JS, Lednicky JA. Cell and molecular biology of simian virus 40: implications for human infections and disease. J Natl Cancer Inst 1999;91:119-34.

Strengthening National Preparedness for Smallpox: An Update

Concern that smallpox virus may be used as a biological weapon of mass destruction has prompted calls for production of additional vaccine and new research into variola virus diagnostics and clinical interventions. Only 15.4 million doses of smallpox vaccine, produced approximately 20 years ago, exist in the United States (1). While virtually all lots remain potent, additional vaccine would clearly be needed in a national emergency involving smallpox virus. Global eradication of natural smallpox disease was declared in 1980; with eradication, most research activities involving the virus ended. Although the complete genomic sequence of selected isolates of variola virus is known (2), the diagnosis and treatment of smallpox infection have not changed in the past two decades. Recognizing the need for advancement in these areas before variola virus stocks are destroyed, the World Health Organization (WHO) passed a resolution (WHA 52.10) in 1999 extending the date of destruction of all remaining variola virus stocks until the end of 2002. The midpoint of this period is an appropriate time to review progress made in vaccine production and variola virus research and to outline the next steps.

Vaccine Production

On September 20, 2000, the Centers for Disease Control and Prevention (CDC) entered into an agreement with OraVax (Cambridge, MA) to produce a new smallpox vaccine. Like the vaccine used to eradicate smallpox, the new vaccine will contain live vaccinia virus; however, it will be produced in cell cultures by modern vaccine production techniques. OraVax will coordinate full clinical testing of the vaccine and submit a licensing application to the U.S. Food and Drug Administration (FDA) for the prevention of smallpox in adults and children. Forty million doses of the new vaccine will be produced initially, with anticipated delivery of the first full-scale production lots in 2004. The agreement calls for sustained annual production through 2020 to replace outdated vaccine and allows for increased production should an emergency arise. The vaccine will be administered with bifurcated needles (also produced by OraVax), which create a localized vaccine "pock" and confer protective immunity. The vaccine will be held in reserve as part of the national stockpile and be released only in the event of a confirmed case of smallpox or when vaccination against vaccinia virus is warranted. The agreement allows OraVax to produce additional vaccine for other markets, including international buyers.

Variola Virus Research

A research plan, implemented at CDC by scientists from both the Department of Defense and CDC and including extensive collaborations with scientists from the National Institutes of Health and other organizations, is being undertaken with WHO concurrence. All work with live variola virus is done under biosafety level 4 containment conditions at CDC. Smallpox virus is officially retained at only two facilities in the world: at CDC in the United States and the State Research Center of Virology and Biotechnology in Novosibirsk, Russia. Research teams from both institutions are coordinating activities to avoid duplication and gain the maximum amount of information possible before final destruction of the virus.

Strain Evaluation

Of 461 isolates in the smallpox virus collection at CDC, 49 were selected for further characterization. These isolates, which included both variola major and variola minor, were selected to represent the greatest diversity in date of collection and geographic region. Of the 49 isolates tested for viability, 45 were successfully recovered, and seed stocks were prepared for subsequent studies. This group of 45 represented isolates from as early as 1939 and as late as the 1970s; all major geographic regions were represented. Study of these isolates is based on three research themes: application of modern serologic and genomic methods in the diagnosis of variola virus disease; determination of candidate antiviral drug activity against this virus; and investigation of the pathogenesis of smallpox infection, especially through the development of a nonhuman primate model to replicate human smallpox infection. The research team carefully outlined all experimental work to be undertaken with variola virus, incorporating suggestions from a peer group of highly qualified external experts from academia and industry; the first set of experiments was

conducted from January to July 2000 in the CDC maximum containment laboratory.

Serologic Assays

Because enzyme immunoassay technology was still in its infancy when smallpox was eradicated, during the first series of experiments, polyclonal and monoclonal antibodies had to be produced for developing enzyme-linked immunosorbent assays to measure variola virus-specific immunoglobulin (Ig) M, IgG, and antigen. These reagents are now being evaluated by prototype assays with inactivated viral antigens. This work will continue for the foreseeable future.

Nucleic Acid-Based Diagnostics

Viral DNA was extracted from all 45 successfully recovered isolates, was purified and inactivated, and is now being examined by restriction fragment-length polymorphism developed by an extended polymerase chain reaction assay that amplifies viral genome into 20 overlapping products of approximately 10 kilobases each. These products cover virtually the entire length of the viral genome and include sequences in essential genes and genes likely needed for pathogenesis. Preliminary results indicate that the data thus generated offer a good low-resolution overview of genetic diversity of variola viruses and are being used to differentiate strains, infer phylogeny, and identify as many as 10 additional variola isolates for complete genome sequencing. Two isolates, Somalia 77 and Congo 70, were specifically suggested by WHO for sequencing, and this work has begun. A dedicated sequence and bioinformatics facility being developed at CDC will be used to undertake this effort and to begin constructing a genomic signature database, not only for smallpox but also, over time, for other pathogens with bioterrorism potential.

Antiviral Drugs

Two hundred seventy-four antiviral drug compounds were screened for activity and therapeutic indices against variola, monkeypox, cowpox, camelpox, and vaccinia viruses by two cell culture assays. Many of these compounds were provided for testing under collaborative arrangements facilitated by an orthopox antiviral research initiative of the National Institute of Allergy and Infectious Diseases. Previous studies identified a nucleoside phosphonate DNA polymerase inhibitor, cidofovir (Vistide), as being active against poxviruses, including variola. In the current trial, cidofovir and its prodrug (cyclic HPMPC) were evaluated against 31 strains of variola, which were selected to cover a wide geographic area and time span. No substantial differences in inhibition among strains were observed, which suggests that cidofovir-resistant strains are unlikely. The in vitro inhibition was further characterized in multiple cell lines to meet FDA requirements. However, another class of antiviral drugs, the *S*-adenosylhomocysteine hydrolase inhibitors, showed considerable variation in the 50% inhibitory dose between variola isolates; this effect should be investigated further.

Two approaches to the development of an oral prodrug of cidofovir yielded compounds with improved antiviral activity. In addition, the current series of experiments identified 27 other compounds, including completely new classes of drugs, that appear to be active against variola and other orthopoxviruses. In fact, 10 compounds had therapeutic indices greater than 200, while cidofovir had indices greater than 10; 3 compounds had therapeutic indices greater than 1,500. When work resumes in early 2001 with live variola virus, we will continue to evaluate these and additional compounds for activity, including analogs designed for oral administration. The most promising compounds emerging from this in vitro testing will be evaluated in animal models, e.g., cowpox and vaccinia in mice and eventually monkeypox virus challenge in nonhuman primates. All promising compounds will be tested against a battery of surrogate orthopox viruses to guide evaluation of new antiviral compounds after variola virus is no longer available.

Animal Models

A major goal of the current research is to define an animal model that faithfully replicates human smallpox. Such a model would be extremely valuable in evaluating candidate antiviral drugs and novel diagnostic assays and in defining the pathogenesis of smallpox. Consequently, two groups of four cynomolgus macaques were exposed to two variola virus strains at a high dose (>10^8 PFU) by the aerosol route. Clear evidence of infection was found; the

animals had transient fevers, perturbations in cytokine titers in serum, and mild exanthemous lesions. A few of the monkeys showed signs of bronchopneumonia, but none died or had disease similar to the classic smallpox seen in humans. Another series of experiments will be undertaken with different variola isolates to confirm these preliminary observations and generate additional clinical material to validate the diagnostic assays under development.

The results of the research now under way, coupled with the promise of renewed production of smallpox vaccine, will better prepare the United States—and indeed the entire world—for the possibility that smallpox virus might be used as a terrorist weapon of mass destruction.

James W. LeDuc* and Peter B. Jahrling†
*Centers for Disease Control and Prevention, Atlanta, Georgia, USA; †United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland, USA

References

1. LeDuc JW, Becher J. Current status of smallpox vaccine. Emerg Infect Dis 1999;5:593-4.
2. Shchelkunov SN, Massung RF, Esposito JJ. Comparison of the genome DNA sequences of Bangladesh-1975 and India-1967 variola viruses. Virus Res 1995;36:107-18.

High-Level Ciprofloxacin Resistance in *Neisseria gonorrhoeae*: First Report from Israel

To the Editor: We report a case of male gonococcal urethritis that persisted despite ciprofloxacin therapy. The isolate was found to be highly resistant (MIC 32 µg/mL).

A 30-year-old man visited his family physician with a 2-day history of urethral discharge and dysuria. The symptoms began 7 days after a single, unprotected orogenital contact with a female hitchhiker. The patient denied exchange of money for the act and reported no other recent sex partners or travel outside Israel. After a urethral swab was obtained for culture, the patient received a single dose of ciprofloxacin (500 mg orally). Growth of *Neisseria gonorrhoeae* was subsequently reported. However, symptoms persisted, and a regimen of doxycycline (100 mg orally twice a day for 10 days) was initiated. After temporary clinical improvement, the patient returned with worsening symptoms: bloody urethral discharge, severe dysuria, edema of the penis, and painful erection. *N. gonorrhoeae* was reisolated from a repeat urethral swab. When a single dose of ceftriaxone (250 mg) was administered intramuscularly, clinical cure was prompt.

Susceptibility testing was performed on the second isolate by using the E-test method (AB Biodisk, Solna, Sweden) on a medium containing GC agar base and 1% defined growth supplement. The MIC of ciprofloxacin was 32 µg/mL, penicillin 1.5 µg/mL, tetracycline 2 µg/mL, and ceftriaxone 0.016 µg/mL. The isolate did not produce beta-lactamase. It was classified as a $CMRNG^{PT}$ phenotype (*N. gonorrhoeae* with chronomosomally mediated resistance to both penicillin and tetracycline).

Gonorrhea was considered a rare disease in Israel in the 1990s: the average annual incidence was 0.89 reported cases per 100,000 population (1). Most laboratories did not carry appropriate media, and susceptibility testing of *N. gonorrhoeae* was not performed routinely. Quinolones and spectinomycin are the antibiotics most commonly used to treat the infection. Nevertheless, we are not aware of any instance of clinical failure following fluoroquinolone therapy. More recently, however, the incidence of gonorrhea has been increasing (2). In response, a surveillance program for monitoring antimicrobial resistance in *N. gonorrhoeae* has been launched.

Fluoroquinolones and cephalosporins became the recommended drugs for treatment of gonococcal infection after penicillin- and tetracycline-resistant *N. gonorrhoeae* appeared (3). Gonococcal strains with reduced in vitro susceptibility to fluoroquinolones (MIC, 0.125 µg/mL to 0.5 µg/mL) were first described in the mid-1980s (4) and are now occurring worldwide (5).

Fluoroquinolone-resistant *N. gonorrhoeae* (ciprofloxacin MIC $\geq$1.0 µg/mL) emerged during the 1990s and became well established in several Asian countries (6). In Japan, the rate of ciprofloxacin resistance increased from 6.6% in 1993-1994 to 24.4% in 1997-1998 (7). More recently, high-level resistance to ciprofloxacin and reports of treatment failure have appeared (8). Strains with ciprofloxacin MICs of $\geq$8.0 µg/mL were first isolated in 1994 (6) and are detected mostly in the Far East. Two cases of gonococcal infection by strains with an MIC of 16 µg/mL were recently reported in the United States (9). Gonococcal resistance to fluoroquinolones is associated with mutations in the genes encoding DNA gyrase (gyrA) and topoisomerase (parC) as well as change in porin permeability and reduced intracellular drug accumulation (6). In view of the increasing resistance to fluoroquinolones, ceftriaxone, cefixime, or spectinomycin is now recommended if an infection was acquired in Asia or other areas with known fluoroquinolone resistance (9).

Our patient reported no travel to the Far East, and his sex partner, who could not be located for follow-up, was not Asian; her travel history was unknown. The mode of transmission of this infection was fellatio. Condoms are often not used in this form of intercourse, even by those who regularly use condoms for genitogenital sex, because of the mistaken belief that infection is not spread through this form of intercourse. It is now well established that oral sex plays an important role in HIV transmission (10), and condoms should be used with any form of intimate sexual contact.

Michael Dan,* Francesca Poch,* Daniel Shpitz,† and Bracha Sheinberg†
*Infectious Disease Unit, E. Wolfson Hospital, Holon, Israel, and †Maccabi Health Services, Rishon-le-Zion, Israel

References

1. The Israel Center for Diseases Control. Notifiable infectious disease in Israel, 1951-1995. Israel: State of Israel: Ministry of Health; 1996. pub. no. 201.
2. District Health Office, Tel-Aviv. Report on emerging issues, July-December 1999. State of Israel: Ministry of Health; Jan 10, 2000.
3. Centers for Disease Control and Prevention. Guidelines for treatment of sexually transmitted diseases. Mor Mortal Wkly Rep 1998;47(RR-1):1-116.
4. Tapsall JW, Shultz TR, Phillips EA. Characteristics of *Neisseria gonorrhoeae* isolated in Australia showing decreased sensitivity to quinolone antibiotics. Pathology 1992;24:27-31.
5. Quinn TC. The impact of antimicrobial resistance on the treatment of sexually transmitted diseases. Infect Dis Clin North Am 1997;11:884-904.
6. Knapp JS, Fox KK, Trees DL, Whittington WL. Fluoroquinolone resistance in *Neisseria gonorrhoeae*. Emerg Infect Dis 1997;3:33-8.
7. Tanaka M, Nakayama H, Haraoka M, Saika T, Kobayashi I, Naito S. Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998. J Clin Microbiol 2000;38:521-5.
8. Tapsall JW, Limnios EA, Thacker C, Donovan B, Lynch SD, Kirky LJ, et al. High-level quinolone resistance in *Neisseria gonorrhoeae*: a report of two cases. Sex Transm Dis 1995;22:310-11.
9. Centers for Disease Control and Prevention. Fluoroquinolone-resistant *Neisseria gonorrhoeae*–San Diego, California, 1997. Mor Mortal Wkly Rep 1998;47:405-8.
10. Dillon B, Hecht FM, Swanson M, Goupil-Sormany I, Grant RH, Chesney MA, et al. Primary HIV infections associated with oral transmission. Presented at the 7th Conference on Retroviruses and Opportunistic Infections, San Francisco, Jan 30–Feb 2, 2000 (Abstract 473).

An Unusual Bacterium Causing a Brain Abscess

To the Editor: Intracranial abscesses are an important cause of illness and death in a neurologic/neurosurgical unit. Early presumptive clinical diagnosis supported by radiologic evidence (computerized axial tomography [CAT] scan and magnetic resonance imaging) is the mainstay of diagnosis (1). Abscess contents are aspirated under stereotaxic guidance and cultured to isolate causative organisms and determine their antibiotic sensitivities. Organisms isolated from brain abscesses are usually streptococci, anaerobic and facultative gram-negative bacilli, staphylococci, or pseudomonads (2).

A 24-year-old male farmer came to us with progressive headache, dizziness, and a low-grade fever of 2 weeks' duration. He had had a pimple on his right cheek approximately 3 weeks before, which had discharged "bluish" pus on forcible evacuation and subsequently healed without treatment. No focal neurologic signs were detected on physical examination. Because an intracranial space-occupying lesion was suspected, a lumbar puncture was withheld. Later, a CAT scan of the patient's head revealed a right-sided temporoparietal space-occupying lesion approximately 3 cm in diameter, suggestive of a unilocular brain abscess. The abscess was needle aspirated under stereotaxic guidance, and the pus was cultured aerobically and anaerobically. After 24 hours of aerobic incubation on MacConkey agar at 37°C, a pure growth of violet-colored colonies appeared, identified as *Chromobacterium violaceum* by the 20E API system (Biomerieux, France).

Other initial laboratory findings were as follows: blood leukocyte count, 16,200 cells/µL (84% neutrophils, 15% lymphocytes, 1% eosinophils); erythrocyte sedimentation rate (Westergren method), 22 mm/hour; C-reactive protein concentration, 96 mg/L; and fasting blood sugar concentration, 5.1 mmol/L. Blood urea and C-reactive protein concentrations after 3 weeks of antibiotic treatment were 4.6 mmol/L and <6 mg/L, respectively.

The organism was sensitive to imipenem and ciprofloxacin and resistant to cefotaxime and ceftriaxone, by the Stokes comparative disk-diffusion antibiotic sensitivity testing method (3). Ciprofloxacin (as lactate) was administered intravenously, 400 mg twice a day, for 4 weeks. Repeated CAT scans, clinical symptoms, and serial C-reactive protein levels indicated rapid regression of the abscess followed by complete cure.

C. violaceum is a gram-negative bacillus present in soil and aquatic environments of tropical and subtropical countries or regions such as Trinidad, Guyana, India, Malaysia, Florida, and South Carolina. It is a bacterium of low virulence, occasionally causing skin

infections and disseminated disease involving multiple organs in immunocompromised patients. In such cases the disease can mimic septicemic melioidosis (4,5).

In this previously healthy patient, infection probably originated from the facial abscess. The patient was negative for HIV antibody (Serodia), had no history of diabetes mellitus or other compromising illnesses, and had no evidence of immunodeficiency. In a previous case of disseminated *C. violaceum* infection in a young patient, postmortem findings revealed numerous cortical infarcts and hemorrhages (6). Our isolate from a brain abscess is yet another case of a relatively avirulent saprophytic microorganism resulting in a deep-seated infection in a well-nourished, previously healthy person.

Dhammika Nanda Atapattu,* Dhammika Priyal Jayawickrama,*† and Vasanthi Thevanesam*
*University of Peradeniya, Peradeniya, Sri Lanka; †General Hospital, Kandy, Sri Lanka

References

1. Mathisen GE, Johnson JP. Brain abscess. Clin Infect Dis 1997;25:763-81.
2. Mandell GL, Bennett J, Dolin R. Principles and practice of infectious diseases. 4th ed. New York: Churchill Livingstone; 1995: p. 887-99.
3. Stokes EJ, Ridgway GL, Wren MWD. Clinical microbiology. 7th ed. London: Edward Arnold; 1993: p. 239-50.
4. Murray PR, Baron EJ, Pfaller MA, Tenover FC. Manual of clinical microbiology. 6th ed. Washington: ASM press; 1995: p. 503.
5. Mitchell RG. In: Parker MT, Duerden BI, editors. Miscellaneous bacteria. Topley and Wilson's principles of bacteriology, virology and immunity, Vol. 2. 8th ed. London: Edward Arnold; 1990: p. 589-91.
6. Ti TY, Tan WC, Chong APY. Non fatal and fatal infections caused by *Chromobacterium violaceum*. Clin Infect Dis 1993;17:505-7.

First Glycopeptide-Resistant *Enterococcus faecium* Isolate from Blood Culture in Ankara, Turkey

To the Editor: Glycopeptide-resistant enterococci infections are a major problem in hospitals. Infection or colonization by vancomycin-resistant enterococci was first reported in France (1) and the United Kingdom (2); since then, these organisms have been reported throughout the world. In Turkey, vancomycin and teicoplanin have been used to treat serious methicillin-resistant *Staphylococcus aureus* and ampicillin-resistant enterococci infections.

We describe the case of an acute myelocytic leukemia patient with vancomycin-resistant enterococci bloodstream infection. This is the first glycopeptide-resistant *Enterococcus faecium* isolate from our hospital and from Ankara, Turkey. The patient had not been cared for at another institution.

A 68-year-old man, hospitalized with acute myelocytic leukemia, had fever episodes during the neutropenia following three courses of remission-induction chemotherapy (daunorubicin+cytosine arabinoside). A combination of antibiotics including vancomycin, ceftazidime (sometimes imipenem), and amikacin was administered with different regimens during the 5 months of hospitalization. Blood, urine, and rectal swab cultures during this period were positive for different *Enterobacteriaceae* spp. but always negative for vancomycin-resistant enterococci. For long-term hospitalizations, our center routinely performs surveillance rectal swab cultures. At the end of month 5, *E. faecium* was isolated from the blood cultures, just 1 day before the patient's death.

The strain was identified by conventional methods, commercial automatic systems (API Strep-Biomerièux, France), and polymerase chain reaction. Susceptibility patterns showed that the isolate was resistant to all antibiotics except ciprofloxacin and levofloxacin. When the E-test was used, MIC levels for vancomycin, teicoplanin, ciprofloxacin, and levofloxacin were 256 µg/mL, 64 µg/mL, 0.75 µg/mL, and 1.5 µg/mL, respectively. *VAN-A1* and *Van-A2* type resistance genes were detected by polymerase chain reaction. Hacettepe University microbiology laboratories confirmed these results (3,4).

After this strain was isolated, 1,266 stool and 176 rectal swab samples were taken from hospital personnel in three sessions ≥1 week apart, and patients were tested for vancomycin-resistant enterococci. Swab cultures from all environmental surfaces (bed rails, bedside commodes, carts, charts, doorknobs, faucet handles) were also examined. We injected all samples with 5% sheep blood agar with vancomycin (6 mg/L); vancomycin-resistant *E. faecium* was not identified in any sample.

This was the first case of high-level vancomycin-resistant enterococci with a class A phenotype isolated from a person in our hospital or in Ankara, Turkey. To prevent the organism's spread, we implemented the recommendations of the Hospital Infection Control Practices Advisory Committee (5).

Ahmet Basustaoglu,* Hakan Aydogan,* Cengiz Beyan,* Atilla Yalcin,* Serhat Unal†
*Gülhane Military Medical Academy, Etlik Ankara, Turkey; †Hacettepe University, Ankara, Turkey

References

1. Leclercq R, Derlot E, Duval J, Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N Engl J Med 1988;319:157-61.
2. Uttley AH, George RC, Naidoo J, Woodford N, Johnson AP, Collins CH, et al. High level vancomycin-resistant enterococci causing hospital infection. Epidemiol Infect 1989;103:173-81.
3. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification of the species level of clinically relevant enterococci by PCR. J Clin Microbiol 1995;33:24-7.
4. Handwerger S, Skoble J, Discotto LF, Pucci MJ. Heterogeneity of the VanA gene clusters in clinical isolates of enterococci from the northeastern United States. Antimicrob Agents Chemother 1995;39:362-8.
5. Hospital Infection Control Practices Advisory Committee (HICPAC). Recommendations for preventing the spread of vancomycin resistance. Infect Control Hosp Epidemiol 1995;16:105.

Antimicrobial-Drug Use and Methicillin-Resistant *Staphylococcus aureus*

To the Editor: We read with great interest the debate on the contribution of antimicrobial selection pressure to changes in resistance in *Salmonella enterica* serovar Typhimurium and the comparison made with methicillin-resistant *Staphylococcus aureus* (MRSA) (1).

We strongly agree with Davis et al. that infection control practices must play a central role in successful MRSA control programs. However, we disagree that the antimicrobial-drug use practices that contribute to the control of MRSA have not been scientifically defined. In a recent review, we identified more than 20 studies on consistent associations, dose-effect relationships, and concomitant variations, all supporting a causal relationship between antimicrobial-drug use and MRSA (2).

Since our review, seven other studies have reported on the contribution of antimicrobial-drug use to MRSA colonization and infection in patients, or to high MRSA rates in health-care settings (3-9). One study reports a decrease in the rate of new MRSA cases after major reduction in antimicrobial-drug use (5). Although a lower number of discharges and a shorter hospital stay recorded during the 2-year postintervention period have been proposed as other explanations (10), the sharp decrease in new MRSA cases after the new antibiotic formulary was implemented (a delay of only a few months) supports the hypothesis that reduced antimicrobial pressure contributed to the decline. Additionally, at the recent 4th Decennial International Conference on Nosocomial and Healthcare-Associated Infections, at least five reports addressed either (a) antimicrobial-drug use and increased MRSA incidence or (b) antimicrobial-drug use as an independent risk factor for MRSA acquisition or for persistent MRSA colonization after mupirocin treatment (11).

When antimicrobial classes are taken into account separately, cephalosporins and fluoroquinolones are often identified as risk factors for MRSA (2-5,8,11). The mechanisms that would explain the participation of these two classes are not fully understood. However, fluoroquinolones directly enhance the expression of high-level oxacillin-resistant *S. aureus* in vitro (11, p.202). Another recent study shows that sub-MIC levels of ciprofloxacin increase adhesion of quinolone-resistant MRSA (12), which could explain persistent MRSA colonization and failure of mupirocin treatment in patients who received a fluoroquinolone (11, p.197). MRSA outbreaks in surgical patients have been controlled by isolating patients and abandoning third-generation cephalosporins for surgical prophylaxis (3). As stated by Davis et al., dissemination of epidemic clones does not necessarily require antimicrobial selection pressure; however, the above studies suggest participation of antimicrobial drugs in MRSA colonization and outbreaks.

Finally, when citing Dutch infection control measures as an example of successful control of MRSA, Davis et al. omit the fact that, among European countries, the Netherlands has the lowest antimicrobial-drug use in primary health

care (13) and one of the lowest in hospitals (14). Similarly, Nordic European countries report both very low MRSA prevalence and antimicrobial-drug use (13,15). In Denmark, the prevalence of MRSA peaked at approximately 18% among all *S. aureus* isolates (and approximately 30% among blood isolates only) at the end of the 1960s, then regularly decreased during the 10 following years. This decrease has been attributed to various interventions, including increasing awareness of hospital hygiene and an intensive campaign to teach physicians the principles of prudent antimicrobial-drug use. Indeed, the decade witnessed a decrease in the use of streptomycin and tetracycline to which these MRSA strains were resistant. However, determining the relative contribution of these interventions to the disappearance of MRSA strains from Denmark has not been possible since all were implemented at approximately the same time. Since the beginning of the 1980s, the percentage of MRSA has remained extremely low, and below 1% among blood *S. aureus* isolates. Except for a very small number of localized hospital outbreaks, Danish MRSA isolates now represent imported cases from countries with high prevalence. To preserve this low level, patients admitted from foreign hospitals are isolated and screened for MRSA carriage. Health-care workers who have been working in foreign hospitals are also screened before working in Danish hospitals. At the same time, both the overall level of antimicrobial-drug use and the fraction represented by broad-spectrum antimicrobial drugs, such as cephalosporins or fluoroquinolones, remain very low in Danish primary health care and hospitals, according to the 1999 report by the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (available from: URL: http://www.svs.dk/dk/Organisation/z/forsider/Danmap%20forsider.htm).

Additional research is certainly needed to fully understand the relationship between antimicrobial use and MRSA. However, the evidence supports implementation of programs to control or improve prescriptions when infection control alone does not control MRSA or the organization and resources for a "search-and-destroy" MRSA control strategy are not available.

Dominique L. Monnet and Niels Frimodt-Møller
Statens Serum Institut, Copenhagen, Denmark

References

1. Davis MA, Hancock DA, Besser TE, Rice DH, Gay JM. Reply to Drs. Angulo and Collignon. Emerg Infect Dis 2000;6:437-8.
2. Monnet DL. Methicillin-resistant *Staphylococcus aureus* and its relationship to antimicrobial use: possible implications for control. Infect Control Hosp Epidemiol 1998;19:552-9.
3. Fukatsu K, Saito H, Matsuda T, Ikeda S, Furukawa S, Muto T. Influences of type and duration of antimicrobial prophylaxis on an outbreak of methicillin-resistant *Staphylococcus aureus* and on the incidence of wound infection. Arch Surg 1997;132:1320-5.
4. Hill DA, Herford T, Parratt D. Antibiotic usage and methicillin-resistant *Staphylococcus aureus*: an analysis of causality. J Antimicrob Chemother 1998;42:676-7.
5. Landman D, Chockalingam M, Quale JM. Reduction in the incidence of methicillin-resistant *Staphylococcus aureus* and ceftazidime-resistant *Klebsiella pneumoniae* following changes in a hospital antibiotic formulary. Clin Infect Dis 1999;28:1062-6.
6. Onorato M, Borucki MJ, Baillargeon G, Paar DP, Freeman DH, Cole CP, et al. Risk factors for colonization or infection due to methicillin-resistant *Staphylococcus aureus* in HIV-positive patients: a retrospective case-control study. Infect Control Hosp Epidemiol 1999;20:26-30.
7. Pujol M, Corbella X, Peña C, Pallares R, Dorca J, Verdaguer R, et al. Clinical and epidemiological findings in mechanically-ventilated patients with methicillin-resistant *Staphylococcus aureus* pneumonia. Eur J Clin Microbiol Infect Dis 1998;17:622-8.
8. Schentag JJ, Hyatt JM, Carr JR, Paladino JA, Birmingham MC, Zimmer GS, et al. Genesis of methicillin-resistant *Staphylococcus aureus* (MRSA), how treatment of MRSA infections has selected for vancomycin-resistant *Enterococcus faecium*, and the importance of antibiotic management and infection control. Clin Infect Dis 1998;26:1204-14.
9. Soriano A, Martínez JA, Mensa J, Marco F, Almela M, Moreno-Martínez A, et al. Pathogenic significance of methicillin resistance for patients with *Staphylococcus aureus* bacteremia. Clin Infect Dis 2000;30:368-73.
10. Rice LB. Editorial response: a silver bullet for colonization and infection with methicillin-resistant *Staphylococcus aureus* still eludes us. Clin Infect Dis 1999;28:1067-70.
11. Abstracts of the 4th Decennial International Conference on Nosocomial and Healthcare-Associated Infections, Atlanta, Georgia, March 5-9, 2000. Infect Control Hosp Epidemiol 2000;21:120-122,124,135. (http://www.slackinc.com/general/iche/stor0200/abs1.pdf for pp. 120, 121, 122, and 124; and http://www.slackinc.com/general/iche/stor0200/abs2.pdf for p. 135).

12. Bisognano C, Vaudaux P, Rohner P, Lew DP, Hooper DC. Induction of fibronectin-binding proteins and increased adhesion of quinolone-resistant *Staphylococcus aureus* by subinhibitory levels of ciprofloxacin. Antimicrob Agents Chemother 2000;44:1428-37.
13. Cars O, Mölstad S, Melander A. Large variation in antibiotic usages between European countries [abstract MoP299]. Clin Microbiol Infect 2000;6(Suppl 1):216.
14. Janknegt R, Oude Lashof A, Gould IM, van der Meer JWM. Antibiotic use in Dutch hospitals 1991-1996. J Antimicrob Chemother 2000;45:251-6.
15. European Antimicrobial Resistance Surveillance System. EARSS Newsletter. Apr 2000 (2). Available from: URL: http://www.earss.rivm.nl/PAGINA/DOC/newslapril2000_arial.pdf

Lack of Evidence for Chloramphenicol Resistance in *Neisseria meningitidis*, Africa

To the Editor: High-level chloramphenicol resistance has been reported in 11 epidemiologically unrelated *Neisseria meningitidis* serogroup B strains in Vietnam and in a single strain in France, all isolated between 1987 and 1996 (1). Resistance was mediated by a chloramphenicol acetyltransferase (Cat) encoded by a *catP* gene homologous to *Clostridium perfringens* transposon Tn*4451*. While used infrequently in industrialized countries, chloramphenicol is often used to treat patients with meningococcal disease in Africa, especially during epidemics, when it frequently becomes the drug of choice because it can be administrated intramuscularly (2).

To evaluate the presence of meningococcal chloramphenicol-resistant isolates in Africa, we assessed the frequency of the *catP* gene in 33 *N. meningitidis* strains of serogroup A from the collection of the Centers for Disease Control and Prevention's Epidemic Investigations Laboratory. The isolates, selected to give the maximum geographic and chronological representation, were collected during 1963 to 1998 from Chad, Egypt, Gambia, Ghana, Niger, Nigeria, South Africa, Tanzania, and Uganda, mostly during outbreaks. Thirteen (39.3%) of the strains were isolated during the 1990s, when chloramphenicol resistance was first described in Vietnam. All isolates were characterized by multilocus enzyme electrophoresis and represented four major electrophoretic subgroups (3,4). Chloramphenicol and penicillin MICs were determined for all isolates, according to the recommendations of the National Committee for Clinical Laboratory Standards, by the broth microdilution method using Mueller-Hinton broth with 5% lysed horse blood incubated in 5% CO_2 (5). All isolates were susceptible to both chloramphenicol (MIC <2 µg/mL) and penicillin (MIC <0.06 µg/mL). In addition, we tested all isolates for the presence of *catP* by polymerase chain reaction (PCR) using primers A, B, C, and D (1). Primers A and B, designed from the sequence of *catP,* amplify a 300-bp fragment only in chloramphenicol-resistant isolates. Primers C and D, designed on the basis of meningococcal sequences flanking the Tn*4451*-like insertion, amplify ~1200-bp fragment in resistant isolates and ~200-bp fragment in susceptible strains. Strain LNP13947 (kindly provided by Marc Galimand) was used as a positive control.

The *catP* gene was not detected in 32 of 33 *N. meningitidis* serogroup A strains. One isolate that was negative with primers C and D tested positive with primers A and B (M2786, Nigeria, 1963), which could suggest that *catP* was present but in a different location in the meningococcal genome. However, the chloramphenicol MIC of that strain was 2 µg/mL (susceptible). Repeated attempts to sequence the A/B amplicon were not successful with either primers A and B or another set of primers internal to primers A and B, implying that only a portion of the *catP* gene was present or (even more likely, given the conserved nature of this gene) that the PCR result was a false positive.

Chloramphenicol resistance was first described in meningococcal serogroup B isolates (1), but only serogroup A strains were included in this study since A is the most prevalent serogroup in Africa. (It accounts for most epidemics in Sub-Saharan regions.) Although our small sample size limited the chances of detecting a rare event, the data suggest that chloramphenicol resistance in Africa is relatively infrequent and that chloramphenicol is still an appropriate agent to treat meningococcal disease.

The acquisition of plasmids encoding Cat, which enzymatically inactivate choramphenicol, is the most common mechanism of resistance in gram-positive and gram-negative organisms.

The *catP* gene has been found on various bacterial chromosomes and conjugative plasmids as part of the transposable element Tn*4451*. This transposon is derivative of the Tn*6338* that contains six genes, the largest of which, *TnpX,* is required for the excision of the transposon in both *Escherichia coli* and *C. perfringens* (6). The Tn*4451* derivative that lacked the functional *TnpX* gene was completely stable in both organisms because it had lost mobility as a result of these internal deletions (6). The finding of *catP* in *N. meningitidis* within such a truncated immobile transposon (1) and the possibility of transfer of this type of resistance among highly transformable organisms such as *Neisseria* spp. are of great concern. Were the transposon to become a stable part of the meningococcal genome, it could potentially be easily exchanged. Interspecies recombination between antibiotic-resistant genes of *N. meningitidis* and commensal *Neisseria* spp. has occurred in penicillin- and sulfonamide-resistant meningococci (7-10). A similar occurrence may be possible for *N. meningitidis* chloramphenicol resistance in Africa or other continents where this antibiotic is routinely used for treatment of patients with meningococcal diseases. Studies have not yet demonstrated the clinical significance of chloramphenicol resistance caused by the *catP* gene in meningococci. However, it is possible that, in developing countries, patients whose illness does not respond to antimicrobial agents may not be detected, or their isolates may not be obtained. Screening a selection of isolates for *catP* may allow early detection of chloramphenicol-resistant strains. Since detection of increasing chloramphenicol resistance could change recommendations for antimicrobial-drug therapy, surveillance for antimicrobial-drug resistance should be encouraged.

Acknowledgment

We gratefully acknowledge Jasmine M. Mohammed for performing chloramphenicol and penicillin MICs.

Maria-Lucia C. Tondella, Nancy E. Rosenstein, Leonard W. Mayer, Fred C. Tenover, Sheila A. Stocker, Mike W. Reeves, and Tanja Popovic
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

References

1. Galimand M, Gerbaud G, Guibourdenche M, Riou JY, Courvalin P. High-level chloramphenicol resistance in *Neisseria meningitidis*. N Engl J Med 1998;339:868-74.
2. World Health Organization. Control of epidemic meningococcal disease. WHO Practical Guidelines. 2nd ed. Geneva: World Health Organization; 1998. p.1-84 (WHO/EMC/BAC/98.3).
3. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol 1986;51:873-84.
4. Caugant DA. Population genetics and molecular epidemiology of *Neisseria meningitidis*. APMIS 1998;106:505-25.
5. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 5th edition: approved standard, M7-A5. Wayne, PA: National Committee for Clinical Laboratory Standards; 2000.
6. Bannam TL, Crellin PK, Rood JI. Molecular genetics of the chloramphenicol-resistance transposon Tn4451 from *Clostridium perfringens*: the *TnpX* site-specific recombinase excises a circular transposon molecule. Mol Microbiol 1995; 16:536-51.
7. Spratt BG, Zhang QY, Jones DM, Hutchison A, Brannigan JA, Dowson CG. Recruitment of a penicillin-binding protein gene from *Neisseria flavescens* during the emergence of penicillin resistance in *Neisseria meningitidis.* Proc Natl Acad Sci U S A 1989;86:8988-92.
8. Sáez-Nieto JA, Lujan R, Martinez-Soares JV, Berron S, Vazquez JA, Vinas M, et al. *Neisseria lactamica* and *Neisseria polysaccharea* as possible sources of meningococcal beta-lactam resistance by genetic transformation. Antimicrob Agents Chemother 1990;34:2269-72.
9. Bowler LD, Zhang QY, Riou JY, Spratt BG. Interspecies recombination between the *penA* genes of *Neisseria meningitidis* and commensal *Neisseria* species during the emergence of penicillin resistance in *N. meningitidis*. J Bacteriol 1994;176:333-7.
10. Oppenheim BA. Antibiotic resistance in *Neisseria meningitidis*. Clin Infect Dis 1997;24(suppl 1):S98-S101.

Iron Loading and Disease Surveillance

To the Editor: We read with interest the article by E. D. Weinberg entitled "Iron Loading and Disease Surveillance" (1). Dr. Weinberg proposes routine population screening of iron values by serum ferritin and transferrin saturation tests. Such screening could provide valuable information for epidemiologic, diagnostic,

prophylactic, and therapeutic studies of emerging infectious diseases. However, population screening for hereditary hemochromatosis, the example Dr. Weinberg uses to illustrate his proposal, should await additional data (2-4). At this time, it is not known how many people with genetic risk or biochemical evidence of iron overload will actually become ill. Therefore, the benefits of screening cannot be weighed against the risks of unnecessary treatment. Moreover, standardized, reliable methods for measuring and diagnosing iron overload are not available.

Without additional data, population screening can actually be detrimental to those at risk for disease. Persons with hereditary hemochromatosis may face discrimination, including difficulties in acquiring health, life, or disability insurance. Already, current blood safety policy makes it difficult for them to donate blood, even though blood donation is unlikely to have negative consequences. In addition, the costs of screening for hemochromatosis are not routinely covered by medical insurance nor has the cost-effectiveness of screening been determined. If routine screening is adopted, tracking of persons who test positive must be developed to ensure that appropriate and continuing follow-up is provided and patient confidentiality is preserved.

The Centers for Disease Control and Prevention recommends testing for persons who have either a close relative with hemochromatosis or who themselves experience the unexplained symptoms compatible with the disease (severe weakness or fatigue; unexplained joint or abdominal pain) or its later complications (liver disease, diabetes, or heart problems; impotence; infertility; loss of menstrual periods) (2,5). Testing to exclude other causes of these medical problems should also be performed. Persons with elevated iron or liver function measures should be monitored by their health-care provider.

Michele Reyes and Giuseppina Imperatore
Centers for Disease Control and Prevention, Atlanta, Georgia, USA

References

1. Weinberg ED. Iron loading and disease surveillance. Emerg Infect Dis 1999;5:346-52.
2. Cogswell ME, Mc Donnell SM, Khoury MJ, Franks AL, Burke W, Brittenham G. Iron overload, public health, and genetics: evaluating the evidence for hemochromatosis screening. Ann Intern Med 1998;129(Suppl 11);971-9.
3. Cogswell ME, Burke W, McDonnell SM, Franks AL. Screening for hemochromatosis. A public health perspective. Am J Prev Med 1999;16:134-40.
4. EASL International Consensus Conference on Haemochromatosis. J Hepatol 2000;33:485-504.
5. Witte DL, Crosby WH, Edwards CQ, Fairbanks VF, Mitros FA. Practice guideline development task force of the College of American Pathologists. Hereditary hemochromatosis. Clin Chim Acta 1996;245:139-200.

Reply to Dr. Reyes

To the Editor: The article noted that nearly 50 microbial genera contain strains that are more pathogenic in iron-loaded than in normal hosts. The article proposed "routine screening of populations exposed to certain diseases" but not routine screening of populations at large. A few examples of current interest include atherosclerosis (*Coxiella* and *Chlamydia*), septicemia (*Capnocytophaga*), Whipple's disease (*Tropheryma*), tuberculosis (*Mycobacterium*), gastric ulcers *(Helicobacter*), hepatitis (hepatitis C), and AIDS (opportunistic pathogens).

Of course, the tissue or cell localization of iron and the possible pathogen must be considered. For instance, *Legionella* multiplies in iron-loaded alveolar macrophages but not in plasma. Thus, it would be expected that persons with untreated hemochromatosis with minimal macrophage iron but with high plasma iron would not be at risk for Legionnaires' pneumonia.

Eugene D. Weinberg
Indiana University, Bloomington, Indiana

Meeting Summary

The 5th International Conference on Legionella Ulm, Germany September 26-29, 2000

Twenty-five years after the first recognized outbreak of Legionnaires' disease, the bacterium *Legionella pneumophila* is clearly established as an enduring agent of pneumonia and respiratory disease. This organism causes an estimated 15,000 cases of pneumonia in the United States each year. Since *L. pneumophila* was first recognized, numerous advances have occurred in our understanding of how this unique intracellular pathogen affects humans and how to control it and prevent disease.

These advances were highlighted at the 5th International Conference on *Legionella,* the first international symposium on this organism since 1992. Sponsored by the University of Ulm, Deutsche Gesellschaft fur Hygiene and Mikrobiologie, Vereinigung fur Allgemeine und Angewandte Mikrobiologie, American Society for Microbiology, and Deutsche Forschungsgemeinschaft, the symposium had more than 200 attendees representing 35 countries. The meeting was held in conjunction with the annual meeting of the European Working Group on *Legionella* Infections.

Scientific sessions covered recent developments in pathogenesis, immunology, ecology, clinical microbiology, epidemiology, surveillance, and prevention. The opening lecture was by Joseph McDade, Editor-in-Chief of *Emerging Infectious Diseases* at the Centers for Disease Control and Prevention, who first identified the bacterium in 1977.

Substantial progress has been made in the areas of pathogenesis, development of policies and guidelines to control legionellosis, and potential alternative biocide treatments to prevent colonization of water systems by the bacterium. *L. pneumophila* survives as an intracellular parasite of protozoa and human macrophages. Researchers presented data that further define the mechanisms by which this bacterium enters the host cell, subverts endosomal-lysosomal pathways, acquires nutrients for multiplication, and escapes the host cell. Investigators have identified many novel genes that constitute an unprecedented model for intracellular pathogens. In addition, sequencing of the *L. pneumophila* genome is now >80% complete, with more than 2,000 open reading frames identified. Information on the genome project is available at http://genome3.cpmc.columbia.edu/~legion/.

The meeting also highlighted two large outbreaks of Legionnaires' disease that occurred in the past year: one associated with a cooling tower at the Melbourne Aquarium and the other with a whirlpool spa at a Dutch flower show. Although substantial progress has been made in implementing guidelines to prevent legionellosis, it remains unclear if such preventive measures can suppress the occurrence of large focal outbreaks such as the ones in Australia and the Netherlands. The use of chlorination, copper-silver ionization, monochloramine, and heat to control the bacterium continues to be the subject of controversy. Several presentations focused on the issue of travel-related legionellosis, with much discussion on the best means of detecting and preventing its occurrence. The meeting hosted a formal debate on the use of copper-silver ionization with a panel of five experts in this area.

The proceedings of the meeting will be published by ASM Press, Washington, DC, in 2001. Chicago will host the 6th International Conference on *Legionella* in 2004.

Conference Announcement

Fourth Annual Conference on Vaccine Research: Basic Science-Product Development-Clinical and Field Studies Hyatt Regency Crystal City, Arlington, Virginia April 23-25, 2001

The conference is sponsored by the National Foundation for Infectious Diseases, in collaboration with the Centers for Disease Control and Prevention; National Institute of Allergy and Infectious Diseases, National Institutes of

Health; International Society for Vaccines; Albert B. Sabin Vaccine Institute; World Health Organization; U.S. Department of Agriculture; and the Center for Biologics Evaluation and Research, Food and Drug Administration.

For information, contact Kathleen Hanrahan, National Foundation for Infectious Diseases, 4733 Bethesda Avenue, Suite 750, Bethesda, MD 20814-5228, USA; telephone: 301-656-0003, x19; fax: 301-907-0878; e-mail: info@nfid.org; URL: http://www.nfid.org/conferences.

Erratum Vol. 6 No. 4

In the article, "Communicating the Threat of Emerging Infections to the Public," by V. Freimuth et al., a reference was inadvertently omitted. On page 342, column 1, first full paragraph, information beginning with the sentence "CDC collaborated with other federal agencies..." and continuing to the end of the paragraph was cited from the following reference.

Smith JY, Finkelstein BL, Govert B, Friedman CR, Gold BD, Swerdlow DC. Health communication campaign to increase awareness of *Helicobacter pylori* and peptic ulcer disease. In: Abstracts of the American Public Health Association 126th Annual Meeting and Exposition 1998; Abstract 3326.

We regret any confusion this omission may have caused.

The Bull (detail), 1647

Paulus Potter's The Bull, a very famous painting in its day, drew crowds to the Mauritshuis Museum in The Hague during the 18th and 19th centuries. The Bull is an example of Dutch realism, one of the most striking features of 17th century Dutch painting. Dutch realism is not (as may be implied by the name) simply a highly accurate representation of reality. For a number of artists, realism meant reproducing closely the way light fell on figures and objects. At first glance, a painting may appear an accurate representation of an object or a scene, but study of other related objects or scenes might have been used to create a better composition. Paintings widely praised for their realism usually involved a great deal of manipulation on the artist's part.

The Bull, with its down-to-earth subject matter, life-sized format, and astonishingly realistic details (flies on the bull's coat, the cow's 'damp' nose), is the epitome of Dutch painting. Until a few years ago, the bull in the painting was assumed to be the depiction of an existing bull; however, wide discrepancies between the different parts of the body suggest otherwise. The drooping dewlap and horns indicate a 2-year-old animal, but the teeth (six of which are permanent) are those of a 3- to 4-year-old bull. The forequarters are very muscular, while the hindquarters are underdeveloped. The fore- and hindquarters are at an angle to the pictorial plane, while the animal's body is parallel to it. By all appearances, Potter composed the bull from a number of preliminary studies of different animals.

The first design for The Bull was much smaller. Seams in the canvas indicate that the painter initially intended to paint only the bull. At a later stage, he attached linen strips on either side and along the top of the original canvas, on which he added other animals, the farmer, and the landscape.

Abstracted from The Mauritshuis Museum Catalog, Den Haag, the Netherlands

Editorial Policy and Call for Articles

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal has an international scope and is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, demography, sociology, and other disciplines. Inquiries about the suitability of proposed articles may be directed to the Editor at 404-371-5329 (tel), 404-371-5449 (fax), or eideditor@cdc.gov (e-mail).

Emerging Infectious Diseases is published in English and features the following types of articles: Perspectives, Synopses, Research Studies, Policy Reviews, and Dispatches. The purpose and requirements of each type of article are described in detail below. To expedite publication of information, we post journal articles on the Internet as soon as they are cleared and edited.

Chinese, French, and Spanish translations of some articles can be accessed through the journal's homepage at www.cdc.gov/eid. Articles by authors from non-English-speaking countries can be made simultaneously available in English and in the author's native language (electronic version of the journal only).

Instructions to Authors

Manuscript Preparation

Follow "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" (Ann Intern Med 1997:126[1]36-47) (http://www.acponline.org/journals/annals/01jan97/unifreqr.htm).

Begin each of the following sections on a new page and in this order: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

Title page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Also provide address for correspondence (include fax number and e-mail address).

Abstract and key words. Avoid citing references in the abstract. Include up to 10 key words; use terms listed in the Medical Subject Headings from Index Medicus (http://www.nlm.nih.gov/mesh/meshhome.html).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Type only on one side of the paper and number all pages, beginning with the title page. Indent paragraphs 5 spaces; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use Courier font size 10 and ragged right margins. Italicize (rather than underline) scientific names when needed.

Electronic formats. For word processing, use WordPerfect or MS Word. Send graphics in native format or convert to .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) formats. The preferred font for graphics files is Helvetica. Convert Macintosh files into one of the suggested formats. Submit slides or photographs in glossy, camera-ready photographic prints.

References. Follow the Uniform Requirements style. Place reference numbers in parentheses, not in superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title in full. List the first six authors followed by "et al."

Tables and figures. Create tables within the word processing program's table feature (not columns and tabs within the word processing program). For figures, use color as needed; send files, slides, photographs, or prints. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced. Place figure keys within the figure.

Manuscript Submission

Include a cover letter verifying that the final manuscript has been seen and approved by all authors.

Submit three copies of the original manuscript with three sets of original figures and an electronic copy (on diskette or by e-mail) to the Editor, Emerging Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS D 61, Atlanta, GA 30333, USA; e-mail eideditor@cdc.gov.

Types of Articles

Perspectives, Synopses, Research Studies, and Policy Reviews: Articles should be approximately 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words) and a brief biographical sketch.

Perspectives: Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases or related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change; human demographics and behavior; technology and industry; economic development and land use; international travel and commerce; and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses: This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. Use of subheadings in the main body of the text is recommended. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text. Photographs and illustrations are encouraged.

Research Studies: These articles report laboratory and epidemiologic results within a public health perspective. Although these reports may be written in the style of traditional research articles, they should explain the value of the research in public health terms and place the findings in a larger perspective (e.g., "Here is what we found, and here is what the findings mean").

Policy Reviews: Articles in this section report public health policies that are based on research and analysis of emerging disease issues.

Dispatches: These brief articles are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome. Dispatches (1,000 to 1,500 words) need not be divided into sections. Provide a short abstract (50 words); references, not to exceed 10; figures or illustrations, not to exceed two; and a brief biographical sketch.

Book Reviews: Short reviews (250 to 500 words) of recently published books on emerging disease issues are welcome.

Letters: This section includes letters that give preliminary data or comment on published articles. Letters (500 to 1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

News and Notes: We welcome brief announcements (50 to 150 words) of timely events of interest to our readers. (Announcements can be posted on the journal web page only, depending on the event date.) In this section, we also include summaries (500 to 1,500 words) of conferences focusing on emerging infectious diseases. Summaries may provide references to a full report of conference activities and should focus on the meeting's content.