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**On the Cover:**
Michelangelo Merisi da Caravaggio (1571–1610). Basket of Fruit (1596) Oil on canvas, 45.92 cm x 64.46 cm Pinacoteca Ambrosiana, Milan, Italy

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# EMERGING INFECTIOUS DISEASES

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# Emerging Infectious Diseases in Mongolia

**John R. Ebright,* Togoo Altantsetseg,† and Ravdan Oyungerel‡**

Since 1990, Mongolia's health system has been in transition. Impressive gains have been accomplished through a national immunization program, which was instituted in 1991. Nevertheless, the country continues to confront four major chronic infections: hepatitis B and C, brucellosis, tuberculosis, and sexually transmitted diseases (STDs). As of 2001, only two cases of HIV infections had been detected in Mongolia, but concern grows that the rate will increase along with the rising rates of STDs and increase in tourism. Other infectious diseases of importance in Mongolia include echinococcosis, plague, tularemia, anthrax, foot-and-mouth, and rabies.

Mongolia, situated in Central Asia between Russia and China, has a population of 2.5 million people and an average population density of 1.5 persons per sq. km. Twenty-seven percent of the country's total population lives in the capital city, Ulaanbaatar. The country is divided into 21 administrative provinces called *aimags* with medical care delivered in each province through a three-tiered system; referral to any of the university joint hospitals in Ulaanbaatar is possible. As of 1997, Mongolia had 25 physicians and 78 hospital beds per 10,000 population, one of the highest ratios in Asia (1–3).

Major political and healthcare changes began in 1990, when Mongolia ceased to be under Soviet control and stopped receiving developmental aid as one of the Eastern Bloc satellites (2). Since that time, its economy has been changing from a centrally planned socialist system to a free market economy with healthcare delivery reflecting that transition (1,2,4–6).

Although progress is being made, Mongolia continues to struggle with poor transportation and communication and limited material (including laboratory) facilities. Financial difficulties remain a major challenge as the country seeks to develop economic self-sufficiency and deliver modern health care to its people (1).

*Wayne State University School of Medicine, Detroit, Michigan, USA; †National Medical University of Mongolia, Ulaanbaatar, Mongolia#; and ‡National Research Center for Infectious Diseases, Ulaanbaatar, Mongolia

This report results from two onsite visits by one of the authors (JRE) in September 1999 and May 2001, which allowed for extensive discussions with infectious diseases physicians of the National Research Center for Infectious Diseases (NRCID) and the National Medical University of Mongolia. In addition, he was able to attend hospital ward rounds and conduct patient examinations at NRCID, the Central Hospital of Oncology, First Clinical Hospital, and Third Clinical Hospital in Ulaanbaatar and to review the laboratory facilities in those hospitals.

NRCID serves as the major university hospital and referral center for tuberculosis (TB) and chronic infectious diseases in Mongolia. It is situated on 7 acres of land in south Ulaanbaatar and consists of 23 brick buildings constructed in 1985 by the Soviet Union. Each building is free-standing and requires health providers to walk outside in order to go from building to building (an important point considering Ulaanbaatar's severe and prolonged winters). As a government facility, NRCID is managed and maintained through government funds. Patients are provided care regardless of their ability to pay, and full-time staff physicians receive government salaries.

The data in the following sections of this report were derived from three sources. The first represents the annual cumulative number of specific infectious diseases, which have a high public health impact in Mongolia. These data were collected monthly throughout the country by healthcare personnel in all rural districts and provinces, as well as in Ulaanbaatar, and reported to the Ministry of Health and Social Welfare. The annual cumulative data for each disease were summarized and published for the medical community and general public by the National Statistical Office (Table). The second source is derived from the personal discussions held with leaders in public health and microbiology as well as infectious disease clinicians and faculty at NRCID, the National Medical University, and other hospitals in Ulaanbaatar. The third consists of recently published reports dealing with infectious diseases and public health issues in Mongolia during the past 10 years and identified through Medline search.

Table. Annual cumulative number of reportable infectious diseases by year, Mongolia[a]

| | Y | | | | | |
|---|---|---|---|---|---|---|
| Disease | 1990 | 1995 | 1996 | 1997 | 1998 | 1999 |
| Tuberculosis | 1,664 | 2,543 | 3,104 | 2,723 | 2,806 | 3,221 |
| Viral hepatitis | 14,278 | 7,877 | 8,198 | 9,394 | 8,042 | 5,249 |
| Brucellosis | — | 850 | 1,158 | 1,122 | 1,308 | 1,482 |
| Syphilis | 705 | 718 | 810 | 1,291 | 1,329 | 1,093 |
| Gonorrhea | 2,234 | 3,308 | 3,274 | 2,934 | 3,486 | 2,207 |
| Salmonellosis | 866 | 360 | 323 | 256 | 239 | 243 |
| Shigellosis | 1,930 | 1,589 | 2,294 | 2,146 | 1,261 | 1,383 |
| Measles | 296 | 558 | 123 | 4 | 8 | 10 |
| Mumps | 240 | 255 | 436 | 736 | 1,287 | 426 |
| Meningococcal meningitis | 776 | 2,781 | 881 | 533 | 303 | 242 |
| Chickenpox | 810 | 401 | 386 | 253 | 375 | 297 |

[a](ref. 9, modified)

## TB

A separate building at NRCID is dedicated to management of patients with TB. Divided by separate floors into pediatric, adult, and surgical units, the TB hospital houses approximately 230 patients and serves as the primary referral and treatment center for Mongolia. Diagnosis is made by clinical symptoms, chest x-ray, Mantoux skin test, and positive acid-fast smears of clinical specimens. Availability of culture and susceptibility data appears to be inconsistent but is expected to improve when the National Tuberculosis Central Laboratory is moved on site at NRCID.

Staff physicians treat all patients with isoniazid, rifampin, pyrazinamide, and ethambutol (or streptomycin) for the first 2 months of a standard 6-month treatment course and administer treatment by direct observation (DOT). This treatment is an improvement compared with the TB program of the mid-1990s, when only 29% of patients received a four-drug treatment course (7). Current data from the pediatric unit indicate that 93% of patients became smear-negative after the initial 2-month DOT program compared with 100% 5 years ago. This finding is similar to results (92% smear-negative) reported from a pilot study that used the same antimicrobial drugs and DOT program in eastern Mongolia in 1999 (8). On becoming smear-negative, the patients are discharged to their homes and complete the remainder of their 6-month program with isoniazid and rifampin alone. Both the central hospital and regional clinics cooperate to oversee the completion of therapy after discharge and to ensure that contact tracing is performed. Anti-TB medication is available only through the central TB hospital and regional clinics and not from private pharmacies. In general, no second-line anti-TB drugs, except ciprofloxacin, are available in Mongolia.

National statistics show the lowest incidence of TB in the past 10 years occurred during 1990 to 1994 (1,664 cases, 1990) with a marked increase by 1995 continuing through 1999 (3,104 cases, 1996; 3,221 cases, 1999) (9). The increase probably represents a reporting bias resulting from a relative lack of evaluation and treatment available during the first few years after Mongolia was no longer under Soviet control and until the national TB program was established in 1994 (7). Data from the National Tuberculosis Central Laboratory in Ulaanbaatar characterize the 3,221 cases that occurred in 1999 as follows: pulmonary, 2,280 cases (70%) with 1,513 diagnosed by positive sputum smears and 767 (33% of total pulmonary cases) diagnosed clinically in spite of negative smears; and extrapulmonary, 941 cases. Susceptibility data available from the same source indicate primary resistance to isoniazid is >10%; streptomycin resistance is >10%; any resistance is 29%; and multiple-resistant isolates is 1.1% (N. Naranbat, pers. commun.).

## Viral Hepatitis

Hospital directors of NRCID as well as senior infectious diseases faculty of the National Medical University agree that the most common reportable infection in Mongolia is viral hepatitis. This fact is still true even though the incidence has decreased from 14,278 cases in 1990 to 5,249 cases in 1999 (9). Information regarding frequencies of specific types of viral hepatitis is more difficult to obtain. Serologic testing for hepatitis C has been readily available only since 1999. One physician working in the NRCID serology laboratory stated that approximately 30% of its current assays on inpatients with acute or chronic hepatitis are positive for hepatitis C. However, most (80%) acute hepatitis cases in Mongolia appear to result from hepatitis A (L. Togooch, pers. commun.). A recent report by Japanese investigators gives seroprevalence rates in 150 outpatients in Ulaanbaatar of 39% (hepatitis B) and 48% (hepatitis C). All participants in this study also had volunteered to participate in an epidemiologic study of hyperlipidemia at the Central Clinic Hospital in Ulaanbaatar (10). The incidence of hepatitis B is falling as a result of two major interventions begun in 1991. At that time, a national immunization program,

including vaccination against hepatitis B, was started. In addition, a program to stop the reuse of phlebotomy needles and needles for injection was begun. Currently, all such needles are sterile, individually packaged, and disposable. Evidence attesting to the effectiveness of these interventions is emerging. Edstam et al. report a drop of hepatitis B carriage from a historical prevalence of 14% to 6.9% in a random cluster sampling of Mongolian 2-year old children. These encouraging results were found in both rural as well as urban settings with one dramatic exception. For unclear reasons, 40% of persons in the rural *aimag* Bayanhongor were positive when tested for hepatitis B surface antigen (11).

In spite of the overall encouraging decline in hepatitis B carriage, the impact of chronic hepatitis remains a major health problem for the country. Hepatocellular carcinoma is the most common malignancy in Mongolia (followed by gastroesophogeal, cervical, and lung cancers), and cirrhosis remains common enough to justify a 54-bed ward at NRCID dedicated to the care of patients with the disease. Although injection drug use appears to be rare, alcohol abuse is very common and almost certainly contributes to Mongolia's problem with chronic liver disease (12).

Infectious disease physicians at the National Medical University and NRCID are well aware of the use of interferon for treatment of chronic hepatitis B and C but have been limited in utilizing it, primarily because of its expense. Only approximately 100 patients had been treated with interferon by 2001.

## Brucellosis

Approximately 23% of Mongolia's population lives in rural areas and leads a nomadic or seminomadic way of life. Their diet is heavily dependent on meat and dairy products, reflecting the importance that large domesticated animals have played in the country's history. In the past decade, the number of livestock has increased from 26 million to over 33 million, including 26 million sheep and goats, 3.8 million cattle, 3.1 million horses, and 350,000 camels (13). Not surprisingly, brucellosis remains one of the major veterinary and public health problems in Mongolia. The Brucella seroprevalence rate among cattle in 1987 ranged from 3.8% to 35% before a vaccination program (14) but now appears to be approximately 5%-10% with some focal areas close to 50% (Andrea Mikolon, pers. commun.). Seroprevalence in sheep and goats is less, approximately 2%. Nevertheless, *Brucella melitensis* appears to be the most common species of Brucella isolated from blood cultures taken from acutely ill patients (Andrea Mikolon, pers. commun.). In Mongolia, transmission to humans occurs primarily from direct contact with animals through injury while handling them or during slaughtering and to a lesser extent, from drinking contaminated milk (Andrea Mikolon, pers. commun.). As of 2001, approximately 8,000 human cases of chronic brucellosis were reported, and 1,000–1,500 new cases have been reported yearly since 1996 (9) (compared with approximately 100 cases annually in the United States [15]). Such a high caseload in this sparsely populated country is reflected in a 50-bed unit at NRCID dedicated to caring for people with this disease. Most patients on that unit appear to have chronic skeletal disease diagnosed by clinical features, x-ray findings, and positive serologic results. Cultures are rarely done because of lack of appropriate safeguards for this level III pathogen, but they may be performed occasionally at the University Central Laboratory of the National Medical University. Confirmation and speciation by using polymerase chain reaction (PCR) may also be obtained at the same facility. Treatment with doxycycline and gentamicin or rifampin is standard on the unit, but they are often administered for only 2 weeks rather than the minimal 6 weeks recommended in most recent reviews (15,16). Possibly, as a result, many of the inpatients have a history of relapsing disease and have been admitted for treatment on multiple previous occasions.

Attempts to control this enzootic infection have been unsuccessful because of an inconsistent strategy varying between vaccination of livestock and the destruction of infected animals. As of 2001, no uniform animal vaccination program existed in Mongolia. However, the World Health Organization has recently been conducting meetings with the Ministry of Health and the National Medical University to further assess the health impact of brucellosis in the country and make recommendations for its control.

## Sexually Transmitted Diseases

Sexually transmitted diseases (STDs) have become an increasing problem since Mongolia became fully independent of Soviet control in 1990. This increase may relate partly to temporary loss of central governmental control as well as a decreased economic base for the country. (The Soviet Union had provided 85% of developmental aid to Mongolia, amounting to 35% of the government's annual budget [2]). Physicians at NRCID estimate that 600–1,100 female prostitutes lived in the country in 2001 and suggest, although data are incomplete, that this number represents an increase over the previous decade.

Reported cases of syphilis ranged from 705 to 810 annually during the years 1990 through 1996, but increased to 1,291 in 1997 and 1,329 in 1998. For most of the past decade, gonorrhea was reported in over 3,000 patients yearly except in 1990 (2,234 cases) and 1999 (2,207 cases) (9). These official statistics coincide with the perception of infectious diseases physicians of NRCID that the STD rate has increased since 1990. In addition, a 1997 report by Purevdawa et al. gives further support for

this concern. The authors state that rates of syphilis, gonorrhea, and trichomoniasis cases per 100,000 population increased from the mid-1980s (or 1993 in the case of syphilis) to 1995 as follows: syphilis, 18 to 32; gonorrhea, 51 to 142; and trichomonas, 47 to 155 (17). A recent study conducted in Ulaanbaatar among 260 patients attending a STD clinic found the prevalence of gonorrhea, chlamydia, and syphilis to be 31.1%, 8.1%, and 8.6%, respectively, for male patients and 10.3%, 9.9%, and 6.0% for female patients. Seventy-seven percent of female patients had trichomoniasis and nearly 20% of male patients had nongonococcal urethritis (18). Similar results were found in 110 women attending an STD clinic in Ulaanbaatar from whom genital samples obtained by insertion and immediate removal of tampons were tested by using PCR amplification. *Chlamydia trachomatis* (14%), *Neisseria gonorrhoeae* (11%), and human papillomavirus (HPV) (36%) were detected in the samples. Forty-four percent of the women with human papillomavirus had oncogenic genotypes detected (19).

In addition, antimicrobial drug resistance has become common in *N. gonorrhoeae* isolates. Forty-eight percent to >50% have been found to be resistant to penicillin; close to 15% are resistant to tetracycline, and nearly 25% are resistant to ciprofloxacin (20,21).

Approximately 20,000 patients have been screened for HIV infection at NRCID every year since 1987. The laboratory uses a microtiter agglutination system obtained from Japan as a rapid screen and enzyme-linked immunosorbent assay (ELISA) from either Japan or Italy as second-level tests on samples found positive by using the screen. As of 2001, two patients were positive: one acquired HIV in another country, and the second is thought to have acquired HIV through contact with an African person visiting Mongolia. Nevertheless, the increased rates of STDs since the mid-1980s, in addition to increased tourism in the country, raise concern that HIV will become a problem in Mongolia and justify the public health education initiatives already taking place.

## Respiratory Tract Infections, Infectious Causes of Diarrhea, and Parasitic Infections

Respiratory tract infections and diarrhea are very common in Mongolia, especially among children. Generally, respiratory illnesses including pneumonia are most common during the winter and, at least in 1991, accounted for most the country's infant mortality rate (2). Officially, the infant mortality rate in 1990 was 64.4 per 1,000 live births and decreased to 37.3 per 1,000 live births in 1999 (22).

Infectious diarrhea, on the other hand, is more problematic during the short summer season. Laboratories in the major hospitals of Ulaanbaatar including NRCID identify enteric bacterial pathogens by using manual methods. The most commonly identified pathogens have been *Shigella flexneri* and *Salmonella* species such as *S. enteritidis* Typhimurium (D. Regzedmaa, pers. commun.). However, supplies and equipment necessary to isolate and identify *Campylobacter* species, enterotoxigenic and enterohemorrhagic *Escherichia coli,* and rotavirus are not available; consequently, information regarding the prevalence of these organisms is not available.

An outbreak of cholera involving approximately 100 persons occurred in 1996 but was rapidly brought under control. No cases have been identified since that time, and the source of the outbreak remains uncertain. Typhoid fever appears to be uncommon. At NRCID, one case has been recognized since 1999. In that instance, a 42-year-old man with fever, diarrhea, and a perforated small bowel required resection after he had treated himself before hospitalization with ampicillin and gentamicin.

Official statistics show a gradual decline in reported cases of salmonellosis and dysentery from 866 and 1,930 cases, respectively, in 1990, to 243, and 1,383 cases in 1999 (9). Nevertheless, infectious diarrhea remains a major health problem during the summer months in Ulaanbaatar and a 34-bed unit at NRCID is dedicated to the care of patients with this disease.

The director of the enteric parasite laboratory at NRCID reported that *Enterobius vermicularis* constituted approximately 90% of detected intestinal parasites. The remainder consisted of *Ascaris lumbricoides* and *Taenia* species. This finding corresponds with a recent survey of rural residents near Ulaanbaatar which found that enterobiasis and hydatidosis were the two major helminthic infections in Mongolia. Lee et al. offered the speculation that soil-transmitted parasites such as *A. lumbricoides* are less common because soil-derived vegetables are not commonly included in the typical Mongolian diet (23).

Most households in rural Mongolia own livestock (sheep, goats, horses, cattle) and over 50% own dogs. Not surprisingly, human infection with *Echinococcus granulosis* is seen by physicians in Ulaanbaatar, although less frequently than in the neighboring northern provinces of China. A recent survey conducted in northwest Mongolia detected a seroprevalence of 5% by using an ELISA on 334 persons (24). An earlier report by Davaatseren et al. claimed echinococcus was the cause for 18% of the surgical cases in the First Clinical Hospital of Ulaanbaatar in 1993 (25).

## Other Infectious Diseases

Cases of plague, due to infection with *Yersinia pestis,* have been seen in approximately 40 patients each year, especially in rural Mongolia. Transmission appears usually to occur as a result of hunting marmots (*Marmota sibirica)*, large rodents especially plentiful throughout the vast

steppes of Central Asia. A recent case of pneumonia occurred in Mongolia's western-most province (*aimag*), Bayan Olgii; the 93 persons who had contact with the patient received prophylactic tetracycline. Enzootic plague also may be maintained in the Mongolian gerbil (*Meriones unguiculatus)* and its flea (*Nosopsyllus laeviceps)* (26).

Occasional cases of tularemia have been reported in Mongolia. Animal cases of anthrax involving cattle, sheep, and goats are reported sporadically in the country. Occasional human cases of cutaneous or intestinal anthrax also have been reported. An animal vaccine is available but has not been administered as part of a nationwide program.

Bovine spongioform encephalopathy is unknown in Mongolia and is not anticipated because all cattle are strictly pasture-fed. On the other hand, foot-and-mouth disease caused by apthovirus of the family *Picornaviridae,* although thought to be eradicated in 1973, reappeared along the Chinese border in the year 2000. More recently, it spread from the eastern Mongolian provinces and by March 2001 appeared among the livestock on the outskirts of Ulaanbaatar. Prompt intervention including livestock vaccination brought the outbreak under control, and most districts around the capital city had their quarantine lifted by May 2001.

Rabies remains an endemic problem especially among dogs and wolves, with occasional human cases reported. Although rabies immunoglobulin and human diploid cell vaccine are not available, a locally produced, goat brain–derived vaccine is available for use in Mongolia.

Lyme disease is not reported in the country, but a recent study in northeastern China (Inner Mongolia) documented *Borrelia garinii* and *B. afzelii* in *Ixodes persulcatus* ticks (27). Therefore, the possibility exists of human disease occurring in Mongolia. Tick-borne encephalitis due to a flavivirus infection and hemorrhagic fever with renal syndrome, both present in Russia, are not recognized in Mongolia (28).

## Antimicrobial Drug Resistance

Antimicrobial drugs generally available at NRCID include penicillin G, ampicillin, oxacillin, cephalosporins, including ceftriaxone and cefotaxime, gentamicin, chloramphenicol, ciprofloxacin, tetracycline, erythromycin, trimethoprim-sulfamethoxazole, rifampin, isoniazid, ethambutol, and pyrazinamide. Aminoglycoside blood levels were not routinely available, as of 2001, although they could be obtained through private specialty laboratories in Ulaanbaatar.

Oral antimicrobial drugs have been preferred over those administered parentally, whenever possible. Sterile intravenous infusion solutions are available and prepared locally at each major hospital in Ulaanbaatar. However, intravenous catheters are not readily available, which necessitates that intravenous antimicrobial drugs be given through sterile needles. These needles are promptly removed after each infusion. As of 2001, antimicrobial drugs are commonly prescribed for at least 3 postoperative days to patients undergoing even minor surgery (such as simple lipoma removal) to reduce the incidence of postoperative wound infections.

Each major hospital in Ulaanbaatar has its own bacteriology laboratory, where organisms are identified by using manual methods and susceptibilities are determined by using antimicrobial drug disks. Nevertheless, antimicrobial drug susceptibility profiles appear to be unavailable. Several hospital laboratories could not, for example, provide information regarding their prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA). The director of the The World Health Organization (WHO)-sponsored PCR section of NRCID central laboratory reported a rate of 20% to 30% MRSA. However, no details or written reports were available for review.

## Hospital Infection Control

Sinks and bar soap are available throughout all hospitals. Individual paper towels were not present, however, and reusable cloth towels were used in their place. Healthcare providers, as a rule, did not wash their hands between examining patients during rounds; rather, hand washing generally was done only at the end of rounds after all patients had been seen.

## Immunization

Mongolia's current national immunization program began in 1991 and includes vaccines against TB, polio, hepatitis B, diphtheria, pertussis, tetanus, and measles. As of 2001, the vaccination rate for all children in the country was 98%. Immunization against rubella was to be added to the official program during 2002. Mumps vaccine is not on the official vaccination schedule, however. Of interest, an outbreak of mumps occurred in Ulaanbaatar during the spring of 2001. Mongolia did not experience the outbreak of diphtheria in the 1990s as Russia did (28). Four cases were reported for the country in 2000. WHO certified that Mongolia was free of polio in 2000. Immunization against influenza is sporatic and dependent on outside funding. (L. Togooch, pers. commun.).

## Summary

Since 1990, Mongolia's health system has been in transition. Impressive gains have been accomplished through their national immunization program, started in 1991. Of particular note is the decreased incidence of acute hepatitis B. Nevertheless, the country continues to struggle with chronic hepatitis B- and C-related liver failure and hepatocellular carcinoma (exacerbated by alcoholism, an addi-

tional common social health problem in Mongolia). Three other major infectious disease problems include brucellosis, TB with significant single drug resistance being identified, and rising rates of STDs. Respiratory illnesses including pneumonia constitute a major cause of infant deaths, especially during the prolonged winters, and infectious diarrhea remains a common public health problem during the summers.

As of 2001, only two HIV infections have been detected in Mongolia. Nevertheless, public health leaders remain concerned that its incidence may soon increase. Other infectious diseases of importance include echinococcus, plague, tularemia, anthrax, foot-and-mouth, and rabies.

Antimicrobial drugs are available to the public without prescription and also may be given excessively to postoperative patients. Antimicrobial drug–susceptibility trends generally are not available at the major teaching hospitals in Ulaanbaatar. Infection control policies, especially handwashing, appear to need more attention.

## Challenges

We conclude with our recommendations emphasizing areas for focus and further development, which would seem most applicable and potentially beneficial over the next 10 years. Further progress in transitioning from the clinical diagnosis of TB to diagnosis confirmed by widespread availability of culture for *Mycobacterium tuberculosis* (with accompanying antimicrobial drug–susceptibility results) will be a great help in directing limited resources and optimizing individual treatment programs by using DOT. Given the high prevalence of chronic viral hepatitis (and for other reasons related to social and economic development issues), a national program that reduces individual excessive alcohol consumption through education and treatment centers is appropriate. A national, consistently applied program to reduce the prevalence of Brucella infection of animal livestock is needed. Continued emphasis on public health measures to reduce the spread of sexually transmitted diseases and prevent the incursion of HIV is of great importance. A national program for control of antimicrobial drug use based upon physician prescription rather than general public access is necessary. A system of consistently performing susceptibility studies on common bacterial isolates with subsequent publication of antimicrobial drug–susceptibility trends, at least in the referral hospitals in Ulaanbaatar, would be an important advance. Hospital infection control, including handwashing between individual patient examinations and antimicrobial drug use policies, needs to be further developed in Mongolia. Expansion of the excellent national immunization program to include rubella, mumps, influenza, and possibly haemophilus vaccines would provide additional benefit to the people of Mongolia.

Dr. Ebright is an associate professor in the Department of Internal Medicine, Division of Infectious Diseases, and director of Medical Education at Wayne State University. His research interests are in clinical infectious diseases and include travel medicine, infectious diarrhea, and endocarditis.

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Address for correspondence: John R. Ebright, Division of Infectious Diseases, Harper University Hospital, 4 Brush Center, 3990 John R, Detroit, Michigan 48201, USA; fax: 313-745-4052; email: jebright@intmed.wayne.edu

# Raccoon Roundworm Eggs near Homes and Risk for Larva Migrans Disease, California Communities

**Gabriel P. Roussere,* William J. Murray,* Caroline B. Raudenbush,* Michael J. Kutilek,* Darcy J. Levee,* and Kevin R. Kazacos†**

The raccoon roundworm, *Baylisascaris procyonis*, is increasingly recognized as a cause of serious or fatal larva migrans disease in humans and animals. We assessed the potential for infection in three northern California communities by determining the density and distribution of raccoon latrines, where transmission primarily occurs, and the prevalence of eggs at private residences. We collected fecal samples from 215 latrines and found that 44%-53% of the latrines contained *B. procyonis* eggs and that 16%–32% contained infective eggs. Among the properties surveyed, 28%-49% harbored at least one latrine that was positive for *B. procyonis* eggs. The latrine densities in these communities were higher than any previously reported. The presence of *B. procyonis* eggs in raccoon latrines was common, widespread, and closely associated with human habitation. Where raccoon densities are high, education of the public and removal of raccoons may be necessary.

The raccoon, *Procyon lotor*, is a free-ranging mammal found throughout urban and rural areas of North America. Raccoons harbor a wide variety of infectious agents and parasites, many of which are zoonotic. One of these, the raccoon roundworm, *Baylisascaris procyonis* (Nematoda: Ascaridoidea) (Figure 1), is a well-known cause of visceral, ocular, and neural larva migrans in humans and other animals (1–3). Fatal or severe central nervous system (CNS) disease from *B. procyonis* has been reported in >90 species of birds and mammals (2); 13 known cases of neural larva migrans were reported in humans, primarily in children <2 years of age (2–11).

The biologic, morphologic, and ecologic characteristics of *B. procyonis* are similar to those of other ascarid parasites of carnivores such as the common canine roundworm, *Toxocara canis* (1–3). Unless an unusually heavy infection occurs in juvenile raccoons, *B. procyonis* causes little or no clinical disease in its natural host. Like several other ascarids of mammals, *B. procyonis* has a direct or indirect life cycle, depending on the age of the definitive host (2). Raccoons become infected in one of two ways: 1) young raccoons become infected by eating eggs during investigative behavior and during feeding and grooming activities with other members of their social group; 2) adult raccoons acquire the infection by ingesting intermediate hosts (rodents, rabbits, birds) infected with the larvae of *B. procyonis* (2). In intermediate hosts, CNS disease develops

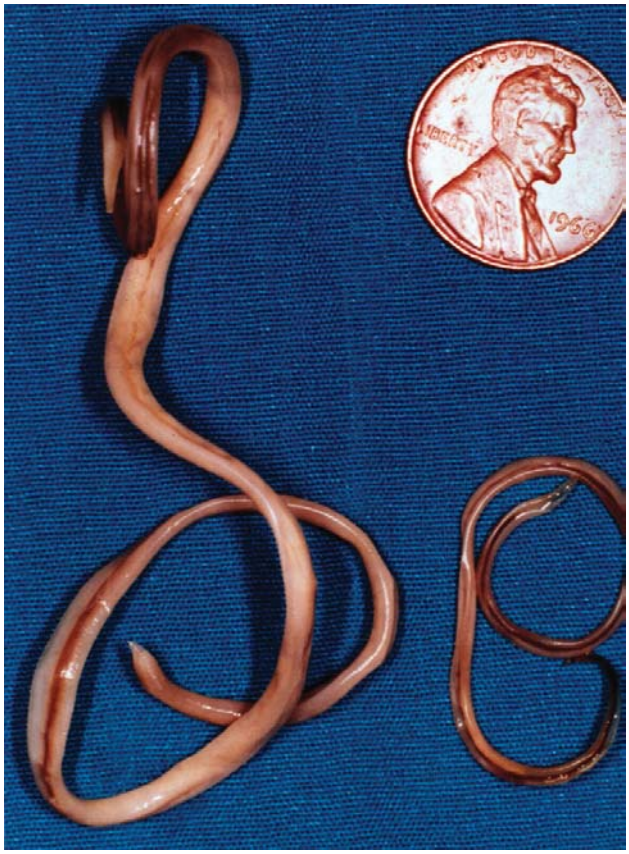

Figure 1. Adult *Baylisascaris procyonis* removed from the small intestine of a raccoon. Adult females (left) are about 24 cm long; males (right) are about 12 cm long. (Reprinted from Clinical Microbiology Newsletter 2002;24:1–7; with permission from Elsevier Science).

*San Jose State University, San Jose, California, USA; and †Purdue University, West Lafayette, Indiana, USA

from larval migration, making the hosts easy prey for raccoons. In either case, the life cycle of the parasite is completed after larvae are released in the intestinal tract and develop into adult male and female worms. This process requires approximately 63 days after egg infection and approximately 35 days after raccoons ingest larvae in intermediate host tissues (2). Adult female worms in the small intestine of a raccoon collectively may produce millions of eggs per day, which are shed in feces (1–3). Once outside the body, the eggs become infective (i.e., contain a second-stage larva) in approximately 2 to 4 weeks, depending on environmental conditions such as moisture and temperature (1–3). Like other ascarid eggs, the eggs of *B. procyonis* are resistant to degradation in the environment and can survive for years under appropriate conditions (1).

A key feature of the epidemiology of baylisascariasis is the behavior of raccoons. Raccoons habitually defecate in communal sites called latrines. The locations of latrines are associated with various natural and human-made structures (2,12). In urban and suburban areas, raccoons establish latrines on rooftops, in attics, in and around chimneys, and on other roof protrusions, stumps, woodpiles, decks, and lawns, especially near trees (1,2) (Figure 2). Where raccoon densities are high, substantial amounts of feces containing large numbers of resistant *B. procyonis* eggs accumulate at latrines, which become long-term focal sources of infection for humans and other animals (1,2,13,14). Thus, humans may become infected accidentally by coming into contact with active or abandoned latrine sites and inadvertently ingesting eggs containing infective *B. procyonis* larvae (Figure 3). Young children are especially at risk for infection because of their propensity to handle objects and put them in their mouth.

Thirteen known cases of *Baylisascaris* encephalitis have occurred in humans in the United States from California, Illinois, Michigan, Minnesota, New York, Oregon, and Pennsylvania. Five of these cases were eventually fatal (4,5,9,11). In addition, a 14th case is suspected in a young girl with CNS larva migrans from Missouri (15). Since 1993, four documented cases of *Baylisascaris* encephalitis have occurred in California, three involving young boys, and one case in a developmentally delayed 17-year-old boy who had pica. Two of the four cases occurred in northern California, in San Leandro in 1993 (7) and Pacific Grove in 1998 (8). The other two cases occurred in southern California, in Los Angeles in 2000 (11) and in Santa Barbara in 2002 (D. Paul et al., unpub. data). In addition, a case of *Baylisascaris* ocular larva


Figure 2. Typical raccoon latrines found in urban/suburban environments. (A) Latrine on a chimney ledge, illustrating the climbing abilities of raccoons and their tenacity in maintaining latrines. (B) Large latrine in the crotch of an oak tree approximately 3.5 m (15 feet) above ground. The sides of the tree were visibly stained with fecal residue that rain had washed down the trunk, contaminating a child's play area below with *Baylisascaris procyonis* eggs. (C) Large latrine, in use for years on a house roof, unknown to the home owner. (D) Latrine site on the ground near downed timber and rocks in a suburban yard. Note the variety of fecal materials (including seeds, crustacean shells, and human refuse), reflecting the diversity of the raccoon diet. The homogeneous-appearing fresh scat in the center is composed of digested pet food. (E) Latrine on a stump in a suburban park with plants sprouting from seeds in the scat. Granivorous birds and mammals are attracted to such locations, as are curious children. (F) Raccoon scat hidden in leaf litter in a suburban back yard, indicating how occult contamination may be.

Figure 3. *Baylisascaris procyonis* eggs recovered from raccoon feces from a latrine in a playground sandbox. Left, infective egg containing a fully formed larva (40x). Right, an undeveloped or degenerate noninfective egg. *B. procyonis* eggs are ellipsoid, approximately 75 µm x 60 µm in size, with a brown, finely granular surface. (Reprinted from Clinical Microbiology Newsletter 2002;24:1–7; with permission from Elsevier Science.)

migrans was identified in a 29-year-old man from Marin County (16).

The present study was prompted by the Pacific Grove case, in which an 11-month-old boy became infected with *B. procyonis* and severe neural larva migrans and unilateral ocular disease (8) developed. Infective *B. procyonis* eggs were found in raccoon latrines that were numerous on the patient's property and an adjacent lot. Further investigation indicated large populations of raccoons throughout the area based on sightings, homeowner complaints, and the presence of raccoon latrines in residential areas. All indicators suggested a substantial potential for transmission of *B. procyonis* to humans and animals. However, despite an increasing recognition of baylisascariasis in humans, data are lacking on the distribution of raccoon latrines and the prevalence of *B. procyonis* eggs in areas where humans reside.

We investigated the risk for exposure to *B. procyonis* in three northern California communities by systematically examining raccoon latrines. The purpose of the study was to determine the density and distribution of raccoon latrines and the prevalence of *B. procyonis* eggs located near human habitation.

## Materials and Methods

### Field Methods

We chose three study areas in northern California that were known to have large populations of raccoons. Pacific Grove and Carmel are coastal cities lying close to one another on the Monterey Peninsula. Both communities have had ongoing complaints from residents regarding raccoon depredation to their homes and properties. A recent documented case of baylisascariasis occurred in Pacific Grove (8), but none occurred in Carmel. The third community, the Naglee Park neighborhood of San Jose, is approximately 112 km (70 miles) northeast of the Monterey Peninsula. It is an inland community lying in the Santa Clara Valley. Naglee Park has had few complaints from residents regarding raccoon activities and no documented cases of baylisascariasis. The three communities are similar in their demographics, and all have older homes and established plantings of mature trees and shrubs.

We used newspaper advertisements and flyers to bring the study to the attention of the residents. Property owners called a telephone messaging system and agreed to have their properties studied. We mapped the locations of the properties on city maps and found that they approximated a random distribution in all three communities. We assigned unique identification codes to the properties, corresponding to the city and the numerical order in which they were studied. We measured the total area of each property in square meters and systematically surveyed each site for the presence of raccoon latrines.

Latrines were identified by the presence of raccoon feces, which have characteristic size, shape, odor, and other physical attributes; typically, they are dark, attenuated scats, approximately 7- to 15-cm long x 2 cm in diameter, have a pungent odor, and contain a variety of seeds and other food items (2,13,17). Fecal piles <1 m apart were considered to be from the same latrine. We mapped the location of each latrine, measured its diameter, and counted the number of scats it contained. All feces were collected from each latrine or, if the volume of feces was large, representative portions of each recognizable scat were collected and placed in either plastic bags or 50-mL plastic tubes. Typically, the fecal weight from a single latrine ranged from 30 g to 750 g. All samples were examined for *B. procyonis* eggs within 1 to 3 days of collection. Samples that were negative were reexamined.

### Laboratory Methods

We determined the presence of *B. procyonis* eggs in fecal samples by a modified detergent wash flotation procedure using Sheather's sugar solution, specific gravity 1.25–1.27 (18,19). In some cases, desiccated samples were rehydrated with distilled water to soften them before processing. Preparations were examined by using a light microscope by bright-field and differential interference contrast methods.

We identified *B. procyonis* eggs (Figure 3) on the basis of their size and other morphologic characteristics (1,2). Once identified, the eggs were classified as noninfective, if they contained an undeveloped embryo, or potentially infective, if they contained a fully formed larva (3).

## Results

We found 127 raccoon latrines on 80 properties in Pacific Grove, 64 latrines on 38 properties in Carmel, and

53 latrines on 46 properties in San Jose, for a total of 244 latrines on 164 properties. Property sizes were more variable in Pacific Grove (1,828 ± 6,023 $m^2$) than in Carmel (777 ± 1,263 $m^2$) or San Jose (752 ± 447 $m^2$). The density of latrines was 8.7/hectare (3.5/acre) in Pacific Grove, 21.7/hectare (8.8/acre) in Carmel, and 15.3/hectare (6.2/acre) in San Jose. In Pacific Grove and Carmel, we found most latrines on roofs (39%-41%); in San Jose most were located on the ground (54%) (Figure 4).

Of the 244 latrines, 29 were not accessible for sampling due to their unsafe locations, e.g., on damaged roofs. We examined the remaining latrines for *B. procyonis* eggs, and approximately half (44.0%-53.2%) of these were positive, with many containing infective eggs (15.9%-31.5%) (Figure 5). Nearly half of the properties in Pacific Grove and Carmel (47.4%-48.8%) and more than one quarter of those in San Jose (28.0%) contained at least one latrine that was positive for *B. procyonis* eggs (Figure 6). Over half of the properties in San Jose (54%) and more than one fourth in Pacific Grove and Carmel (27%-28%) did not harbor latrines. Most of the properties examined had three or fewer latrines, but a few contained as many as eight (Figure 7).

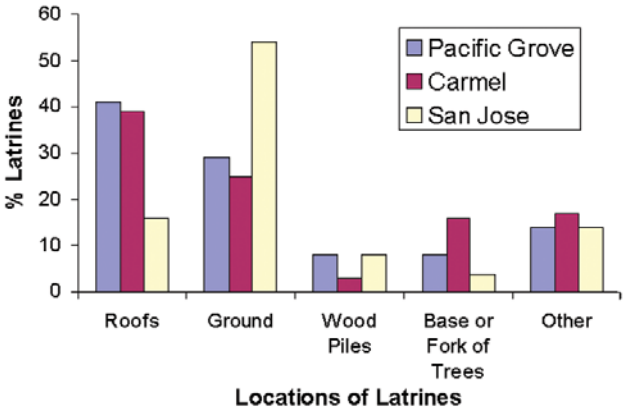


Figure 4. Percentage of raccoon latrines found at various locations in Pacific Grove, Carmel, and San Jose, CA (number of latrines = 244). The "other" category includes window ledges, attics, fences, decks, and so forth.

## Discussion

The widespread distribution and high densities of latrines suggest that human and animal contact with raccoon feces and *B. procyonis* eggs is likely to be common in the study areas. However, human clinical infections are uncommon, probably because most persons do not have intimate contact with the sites or exhibit those behaviors (ingestion, pica, geophagia) that would result in heavy infection. Nonetheless, the potential for infection exists, especially for young inquisitive children who might exhibit such behaviors. Moreover, because of the widespread distribution of raccoons, as well as diagnostic difficulties and lack of clinical experience with the spectrum of disease caused by *B. procyonis* in humans, mild or subclinical infections likely go unrecognized. In fact, asymptomatic, low-level infection with *B. procyonis* is probably the most common form of infection (1,2,3,6).

These study sites had greater densities of latrines and a higher percentage of latrines containing *B. procyonis* eggs than seen in previous studies. Page et al. (12) found 42 latrines in an 8.2-hectare woodlot (5.5/hectare) in Indiana and *B. procyonis* eggs in 14% of the latrines sampled. Jacobson et al. (20) found 97 raccoon scats in a 280-hectare urban study area and 121 raccoon scats in a 230-hectare rural study area in Indiana (0.35 and 0.53/hectare, respectively). They also found that 27% of the urban scats and 31% of the rural ones contained *B. procyonis* eggs (20).

The latrine densities found in our study sites suggest an abundant raccoon population. This conclusion is further supported by field observations of large numbers of raccoons encountered during the surveys. As raccoon density increases, opportunities for intra- and interspecific density-dependent disease transmission also increase. Intraspecific transmission occurs when *B. procyonis* eggs pass from one raccoon to another through social interactions, such as living in the same den and grooming, and by visits to latrines. Interspecific transmission involves intermediate hosts; small granivorous rodents and birds routinely ingest roundworm eggs as they forage for seeds contained in raccoon feces at latrines (2,13,14). The possibility of transmission increases with higher latrine density.

Urban environments typically provide increased food sources and den sites and reduced hunting and predation, leading to higher densities of raccoons (21,22). Anthropogenic food sources, some inadvertent and others purposefully provided by residents, are important con-

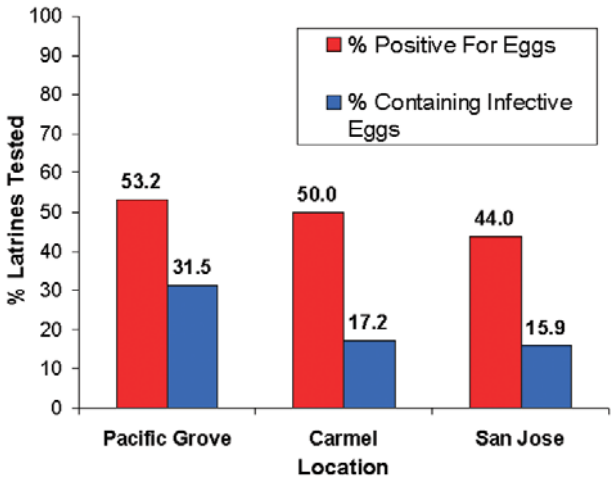


Figure 5. Percentage of raccoon latrines that tested positive for *Baylisascaris procyonis* eggs and those containing potentially infective eggs (number of latrines = 215).

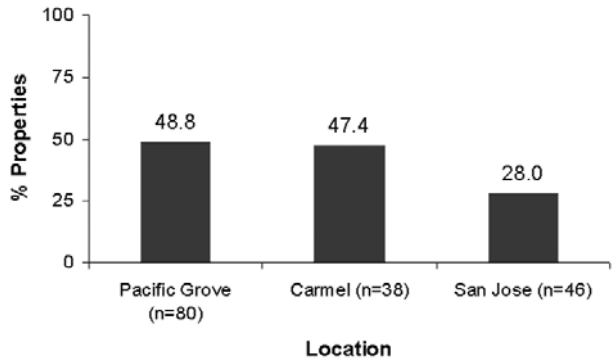


Figure 6. Percentage of properties that contained at least one raccoon latrine positive for *Baylisascaris procyonis* eggs (number of properties = 164).

tributing factors to high raccoon densities. Pet food, which is high in protein and fat content, is an important food source for urban and suburban raccoons. In addition, raccoons may forage on fruits and vegetables from gardens and on food wastes from garbage containers (22). Numerous potential den sites are found in urban areas, including ground-based decks, crawl spaces under homes, outbuildings, culvert pipes, and chimneys. In the Monterey Peninsula cities (Pacific Grove and Carmel), we observed that mature Monterey pines (*Pinus radiata*) were commonly used as daytime resting sites by urban raccoons.

In rural environments and wooded parks, raccoons tend to prefer raised horizontal surfaces, such as logs, stumps, limbs, and forks of trees as latrine sites (1,2,12). Our results show that accessible roofs of houses and sheds are also common locations for latrines in urban/suburban areas. Many roof latrines in Pacific Grove and Carmel were apparently used for years without the homeowner's knowledge. These often accumulated substantial amounts of feces. During periods of rainfall, this fecal matter washes down rain gutters to the ground near residences, thus becoming more accessible to family members. Such ground contamination from roof top latrines has been implicated in a recent case of *B. procyonis* neural larva migrans in an 11-month-old child at a day-care facility in Santa Barbara, California (D. Paul et al., unpub. data).

Ground latrines, which were especially common in San Jose, are also important foci of *B. procyonis* eggs due to their accessibility. Young children (1–4 years of age) frequently have pica or geophagia and often put objects found on the ground in their mouth. They are especially at risk of accidentally ingesting *B. procyonis* eggs and account for most cases of severe infection (2,3). Moreover, feces on the ground decompose into the surrounding soil, leaving no sign of their presence, but infective eggs released from the feces may remain viable for years (1,2).

In the late 1990s, both Pacific Grove and Carmel had ongoing raccoon problems, as judged by frequent complaints from citizens. In Pacific Grove, this situation prompted (since 1998) an ongoing program to trap and euthanize nuisance raccoons. Carmel has had no such programs, which may be the reason for the difference in latrine densities between these two cities. Both Pacific Grove and Carmel have increased their educational efforts aimed at reducing anthropogenic food sources and shelter for raccoons. The study area in San Jose had few complaints, yet the mean number of latrines per property and the mean density of latrine sites was similar to Pacific Grove and Carmel. The major difference was in the distribution of latrines; San Jose had a greater percentage of properties without latrines, compared with the other two cities, suggesting differences in raccoon density and property usage in this community.

*B. procyonis* can produce devastating neurologic disease in humans, especially young children (2–11). *B. procyonis* is much more virulent than the dog roundworm, *T. canis*, the most frequently encountered cause of larva migrans in humans (3). Clinical signs of CNS infection with *B. procyonis* may develop as soon as 2–4 weeks after ingestion of infective eggs (23). Factors influencing the severity of CNS disease in humans and other animals include the number of eggs ingested, the extent and pattern of larval migration in the tissues, especially the CNS, the severity of inflammation caused by migrating larvae, and the amount of tissue necrosis.

Symptoms of baylisascariasis range from varying degrees of mild CNS dysfunction, to severe neural deficits with paralysis, coma, blindness, and death (2–11,23). The larvae of *B. procyonis* have a noted tendency to invade the brain and eye, causing neural larva migrans and ocular larva migrans. Clinical neural or ocular larva migrans is an accidental consequence of somatic migration and larval distribution. Experimental infections in animals have shown that 5%-7% of larvae enter the CNS, but the damage they cause is extensive, attributable primarily to their large size and aggressive migration (1–3,23). Obviously, the greater the number of *B. procyonis* eggs ingested, the more severe the potential clinical problems.

Ingestion of large numbers of *B. procyonis* eggs may produce rapidly fatal neural larva migrans. Despite treatment, progressive neurologic deterioration often continues because of the severe CNS damage and inflammation (2,3,23). Those who survive often have profound neurologic impairment and are severely incapacitated. Their condition may progressively worsen as the brain undergoes postinflammatory atrophy (3,7,23). Diagnosis is often delayed because *B. procyonis* infection is generally not considered during early patient evaluation. This delay contributes to the severity of disease because the larvae continue to migrate unimpeded in CNS tissues, making the prognosis poorer despite later treatment. This infection

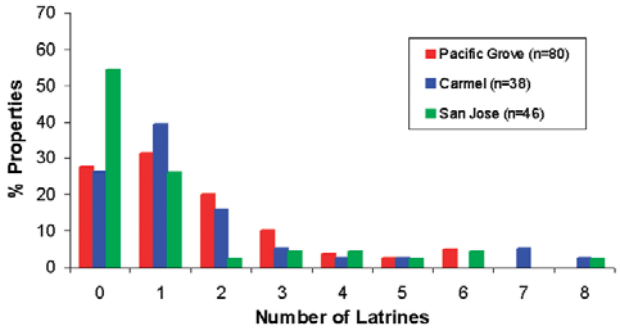


Figure 7. Frequency distributions for the number of raccoon latrines found in Pacific Grove, Carmel, and San Jose, California.

should be strongly considered in any patient, particularly a young child who has eosinophilic encephalitis, peripheral eosinophilia, and a history of possible exposure. Albendazole and steroids should be administered immediately while serologic and other confirmation of *B. procyonis* infection are sought (2,3,7–11).

Human exposure to *B. procyonis* eggs must be prevented, especially in urban and suburban areas where humans and raccoons coexist. Children should be watched carefully when playing in areas with known populations of raccoons and latrines (3). If children are seen to ingest raccoon feces from a latrine or other area, albendazole should be administered immediately (25–50 mg/kg/d x 10 d; or 400 mg twice a day x 10 d), and the raccoon feces sent for examination for *B. procyonis* eggs (3). Children should be taught to avoid fecal material, especially from raccoon latrines, and to thoroughly wash their hands after playing outdoors (3). Property owners are advised to inspect their properties and homes periodically, including roofs, for latrines.

If latrines are found, determining what is attracting raccoons to the location is important. Raccoons are readily attracted to sources of food, water, and shelter. Misguided persons who actively feed raccoons or leave pet food outdoors may contribute greatly to problems for other property owners in an area. Advice can usually be sought from local animal control or wildlife management agencies to determine what is attracting raccoons to the area and how to take measures to exclude them from taking up residence or establishing latrines in a particular location.

Latrines should be removed promptly and fecal material disposed of properly (2,3). Wearing rubber gloves, protective overalls, and rubber boots will reduce the possibility of exposure through self-contamination during cleanup activities. If one is working in a confined space such as an attic, a particle facemask should be worn to reduce the possibility of exposure to fungal spores or other contaminants. Feces should be carefully removed, double-bagged in plastic garbage bags, then placed in routine garbage containers for disposal in a landfill or by incineration. If the latrine is located on the ground, approximately 5–7.5 cm of underlying soil should also be removed and discarded.

*B. procyonis* eggs are difficult to destroy without resorting to high heat (e.g., propane gun flame, boiling water, steam) (2). Obviously, using flame sources around a home is hazardous and should be discouraged unless surfaces like concrete or soil are to be decontaminated. Furthermore, the eggs have a sticky proteinaceous coat that allows them to adhere to surfaces. They can be rendered less sticky by applications of hot water and bleach, which may be useful for removing residual eggs from flammable surfaces. Additional information regarding latrine removal and decontamination can be found elsewhere (2), or by contacting appropriate government agencies. Further studies are needed concerning the survivability of *B. procyonis* eggs under varying conditions and the assessment of optimal, situation-specific methods for inactivation.

In areas where raccoon density is high, trapping and removing raccoons may be necessary to decrease depredation and the accumulation of *B. procyonis* eggs in the environment. Municipalities should educate the public about this parasite and the negative effects of providing food and shelter to raccoons and other wildlife. Reducing the availability of anthropogenic food sources is important to decrease populations of raccoons in urban and suburban environments. Coordinated, comprehensive wildlife management practices are recommended. Given the potential for human exposure to this parasite and considering the risk for very young children, the public should be encouraged to adopt practices that will reduce the possibility of infection by *B. procyonis*.

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Mr. Roussere received his master's degree in biological sciences from San Jose State University in 2001. His research interests include the ecology of wildlife and zoonotic diseases.

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Address for correspondence: William J. Murray, Department of Biological Sciences, San Jose State University, San Jose, CA 95192, USA; fax:408-924-4840; email: murray@email.sjsu.edu

# Global Distribution of Rubella Virus Genotypes

**Du-Ping Zheng,*[1] Teryl K. Frey,* Joseph Icenogle,† Shigetaka Katow,†‡ Emily S. Abernathy,*† Ki-Joon Song,§ Wen-Bo Xu,¶ Vitaly Yarulin,# R.G. Desjatskova,# Yair Aboudy,** Gisela Enders,†† and Margaret Croxson,‡‡**

Phylogenetic analysis of a collection of 103 E1 gene sequences from rubella viruses isolated from 17 countries from 1961 to 2000 confirmed the existence of at least two genotypes. Rubella genotype I (RGI) isolates, predominant in Europe, Japan, and the Western Hemisphere, segregated into discrete subgenotypes; international subgenotypes present in the 1960s and 1970s were replaced by geographically restricted subgenotypes after ~1980. Recently, active subgenotypes include one in the United States and Latin America, one in China, and a third that apparently originated in Asia and spread to Europe and North America, starting in 1997, indicating the recent emergence of an international subgenotype. A virus that potentially arose as a recombinant between two RGI subgenotypes was discovered. Rubella genotype II (RGII) showed greater genetic diversity than did RGI and may actually consist of multiple genotypes. RGII viruses were limited to Asia and Europe; RGI viruses were also present in most of the countries where RGII viruses were isolated.

Rubella virus infection during the first trimester of pregnancy can lead to severe birth defects (congenital rubella syndrome) (1). Live attenuated vaccines, available since the late 1960s (2), are currently in use in roughly half of the countries in the world, including all industrialized countries, although vaccine coverage varies widely (3). Concentration on comprehensive rubella vaccination has recently increased in developing countries in conjunction with measles elimination efforts, particularly in Latin America (4,5). As part of the surveillance component of these efforts, an understanding of the worldwide molecular epidemiology of rubella virus, which is limited, is necessary.

*Georgia State University, Atlanta, Georgia, USA; †Centers for Disease Control and Prevention, Atlanta, Georgia, USA; ‡National Institute of Infectious Diseases, Tokyo, Japan; §Korea University, Seoul, Korea; ¶Chinese Centers for Disease Control and Prevention, Beijing, China; #Institute of Viral Preparations, Moscow, Russia; **Chaim Sheba Medical Center, Tel-Hashomer, Israel; ††Institute for Virology, Infectiology and Epidemiology, Stuttgart, Germany; and ‡‡Auckland Hospital, Auckland, New Zealand

Rubella virus is an RNA virus that is the sole member of the *Rubivirus* genus, within the *Togaviridae* family (6). The rubella virus genome is ~10,000 nucleotides and encodes five protein products, including three virion proteins: the C or capsid protein and two envelope glycoproteins, E1 and E2. The E1 gene sequence has been used for genotyping and phylogenetic analysis of rubella virus isolates (7–10). Thus far, rubella viruses from Europe, Asia, and North America have been found for the most part to group in a single genotype (Rubella Genotype I or RGI) that has a maximum diversity at the nucleotide level of ~5%. However, a limited number of viruses from Asia (China and India), and more recently Italy, formed a distant phylogenetic branch, differing from RGI viruses by 8% to 10%, which was designated Rubella Genotype II (RGII) (8,9,11,12). These two genotypes belong to the single rubella virus serotype (11). Because of limited sampling, the geographic range of RGII has not been determined.

This study was designed to increase information and understanding on worldwide molecular epidemiology of rubella virus. We have performed combined phylogenetic analysis on viruses from earlier studies (8–10) and, to gain further information on RGII viruses, we included viruses collected from the Eastern Hemisphere, namely Russia, South Korea, China, New Zealand, and Israel.

## Materials and Methods

### Rubella Isolates and Sequences

A total of 103 rubella virus E1 gene nucleotide sequences were used in this study; "new" sequences not reported in previous studies are shown in Table 1, a complete list is available online (http://www.cdc.gov/ncidod/EID/vol09no12/03-0242.htm#table1). The length of the sequence was 1179 nt, which covered 8291–9469 nt

[1]Current address: Respiratory and Enteric Viruses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA.

Table 1. "New" rubella virus E1 gene sequences used in this study[a]

| Isolate | Isolation site and y | GenBank no. |
|---|---|---|
| China | | |
| AH2/AH-CHN99 | Anhui, China 1999 | AY326350 |
| AH5/AH-CHN99 | Anhui, China 1999 | AY326351 |
| Germany | | |
| BCM/-GER91 | Germany 1991 | AY326341 |
| G696/-GER98 | Germany 1998 | AY326342 |
| Israel | | |
| I11/TA-ISR68 | Tel Aviv, Israel 1968 | AY326335 |
| I19/HF-ISR72 | Haifa, Israel 1972 | AY326338 |
| I9/JS-ISR75 | Jerusalem, Israel 1975 | AY326334 |
| I13/BB-ISR79 | Bene-Berak, Israel 1979 | AY326336 |
| I15/JF-ISR78 | Jaffa, Israel 1978 | AY326337 |
| I34/TB-ISR88 | Tiberias, Israel 1988 | AY326339 |
| I76/EV-ISR92 | Ein-Vered, Israel 1992 | AY326340 |
| India | | |
| MTS/-IND00 | India 2000 | AY326343[b] |
| Japan | | |
| J05/TK-JPN93 | Tokyo, Japan 1993 | AB072382 |
| J91/GM-JAP94 | Gunma, Japan 1994 | AB072384 |
| J86/ST-JAP95 | Saitama, Japan 1995 | AB072387 |
| J13/HS-JAP97 | Hiroshima, Japan 1997 | AY397695 |
| Korea | | |
| AN1/SO-KOR95 | Seoul, Korea 1995 | AY326345 |
| AN3/SO-KOR96 | Seoul, Korea 1996 | AY326346 |
| AN5/SO-KOR96 | Seoul, Korea 1996 | AY326347 |
| AN6/SO-KOR96 | Seoul, Korea 1996 | AY326348 |
| New Zealand | | |
| JC1/AL-NEZ81 | Auckland, NZ 1981 | AY326331 |
| JC2/AL-NEZ91 | Auckland, NZ 1991 | AY326332 |
| JC5C/AL-NEZ91 | Auckland, NZ 1991 | AY326333 |
| Russia | | |
| C4/MO-RUS67 | Moscow, Russia 1967 | AY247015 |
| C19/MO-RUS68 | Moscow, Russia 1968 | AY247016 |
| C44/MO-RUS69 | Moscow, Russia 1969 | AY247017 |
| C68/MO-RUS73 | Moscow, Russia 1973 | AY247018 |
| C74/MO-RUS97 | Moscow, Russia 1997 | AY247019 |

[a]New E1 gene sequences not previously reported in earlier studies (8-12) are listed in this Table. A complete list of sequences used in this study is available online (http://www.cdc.gov/ncidod/EID/vol09no12/03-0242.htm#table1).
[b]Isolated in Seattle, WA, USA.

of the rubella virus genome (the complete E1 gene is between nts 8258–9700 [13]). This collection consisted of sequences from new isolates from China, Israel, Japan, Korea, New Zealand, and Russia and representative sequences from previous studies (8–10). Methods of isolate propagation and E1 gene sequence determination were as previously described (9).

### Genetic Sequence Similarities and Distances

Distance matrix tables were computed by using the Old distance with Simple correction method in the GCG software package (Wisconsin package version 10.0, Genetics Computer Group, Madison, WI). These tables were used to calculate the average genetic distance among viruses in a single genotypic group or between viruses in two genotypic groups.

### Phylogenetic Analysis

The phylogenetic trees (Figures 1 and 2) were made using Tree-Puzzle 5.0 (14) (maximum likelihood [ML] criterion with 10,000 or 25,000 quartet puzzling steps and HKY85 model of substitution [15]) and viewed by using TreeView (16). To test the consistency of branching, additional software packages were used to construct trees, including PAUP (David L Swofford. 2001. ver 4.0, Sinauer Associates, Sunderland, MA) and PHYLIP (Joseph Felsenstein, ver 3.6α, July 2000, WA). Both ML and maximum parsimony (MP) algorithms in these packages were used. A histogram of the ML distances computed by Tree Puzzle 5.0 (14) was constructed with Excel (Microsoft, Redmond, WA).

## Results

The sequences used in this study consist of representative sequences from three earlier studies (Europe, North America, and Asia, 1961–1997 [8]; Italy, 1991–1997 [9]; and the United States, 1997–2001 [10]) and sequences of viruses collected in Russia, South Korea, New Zealand, Israel, China, Japan, and the United States that have not been reported (these new sequences are listed in Table 1; a complete sequence list is available online (http://www.cdc.gov/ncidod/EID/vol09no12/03-0242.htm#table1). The sequences from the three earlier studies have not previously been amalgamated into a single phylogenetic study. Phylogenetic trees produced with different programs yielded the same major branches, and only representative trees are shown (Figure 1). Branches were defined as reproducible clusterings of sequences by these different programs.

Most of the new sequences belonged to RGI, which emanate from a single node in the tree shown in Figure 1. For simplicity, the tree shown in Figure 1A contains only half of the RGI sequences; the RGI node with all of the RGI sequences is shown in Figure 1B. New sequences belonging to RGI included both from China, all three from New Zealand, all four from Japan, five of six from Israel, two of four from South Korea, and one of five from Russia. Additionally, all of the sequences from the U.S. study (10) and 19 of 21 from the Italy study (9) were RGI. Fourteen sequences were grouped on branches removed from the RGI node, including the three viruses originally used to define RGII, two RGII viruses isolated from Italy (1994) [9], and 9 of the new sequences: 4 from Russia (3 from 1967 to 1969 and 1 from 1997), 2 from South Korea (1996), 1 from Israel (1968), 1 from China (1979), and 1 isolated in the United States from a case contracted in India (2000).

### RGI Viruses

The sequences of viruses isolated before 1980 are colored black in the expanded RGI node (Figure 1B). These

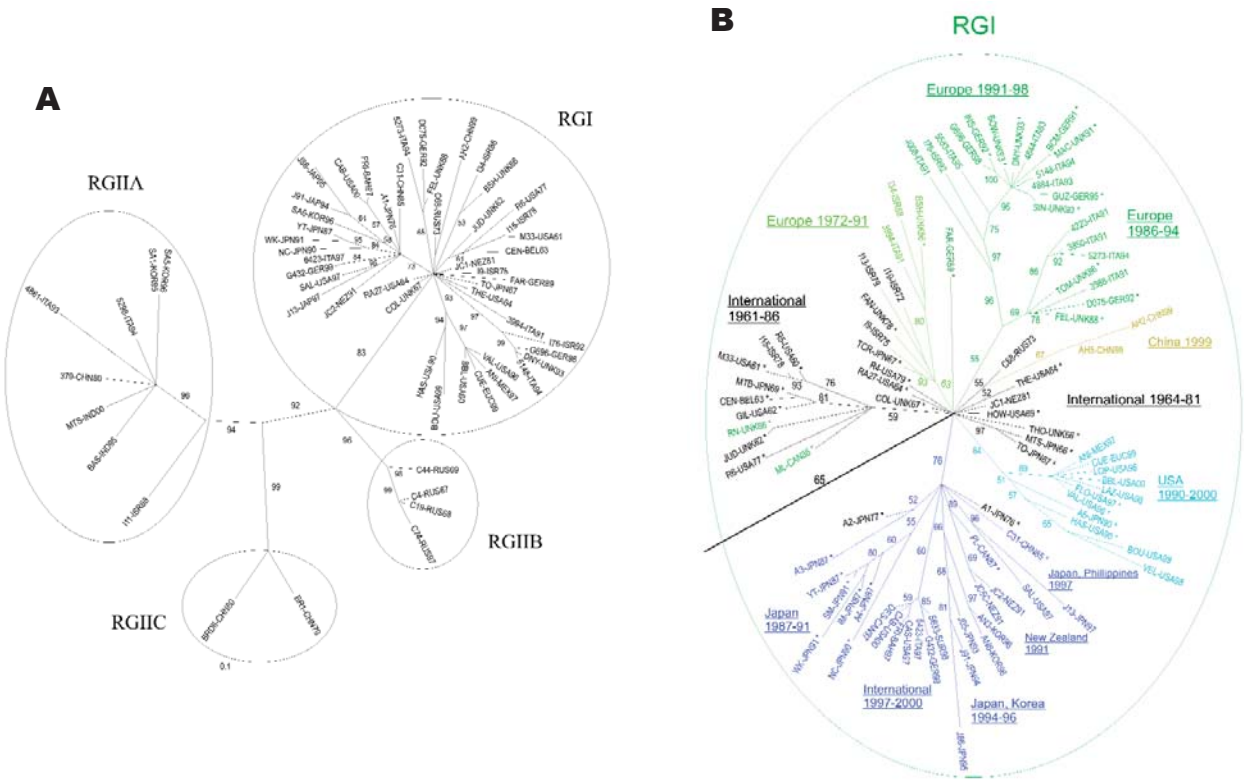


Figure 1. Phylogenetic trees. Unrooted tree was made by the maximum likelihood method in the Tree-Puzzle 5.0 program (25,000 puzzling steps for the tree in A; 10,000 puzzling steps for the tree in B) using the complete E1 gene sequence (1179 nt). Bootstrapping values (out of 100) for each node are given. The tree in A was constructed with half of the rubella genotype I (RGI) and all of the RGII sequences (to allow the reader to read the RGI virus designations); the tree in B is a blowup of the RGI node from a tree constructed with all of the sequences. In B, sequences used in the previous study (8) are designated by an (*), and sequences of viruses isolated before 1980 are in black. Branches are color-coded as follows: RGI International 1961–1986 and 1964–1981, black; RGI Europe 1972–1991, light green; RGI Europe 1986–1994 and Europe 1991–1998, green; RGI China, 1999, gold; RGI USA, 1990–2000, light blue; and a branch containing sub-branches from Japan 1987–1991, International 1997–2000, Japan, Korea 1994–1996, New Zealand, 1991, and Japan-Philippines, 1997, dark blue. Of these, the black International, green Europe, light-blue USA, and dark-blue branches were recognized in the previous study (the light-blue branch as US-Japan and the dark-blue branch as Japan-Hong Kong).

sequences, which were from viruses isolated in Europe, North America, and Asia, congregated around the central node (International 1964–1981) or segregated into one of two branches, denoted International 1961–1986 and Europe 1972–1991. Many of these sequences were used in our previous study (8; marked with an asterisk) in which the sequences in the International 1961–1986 branch formed three related branches, the sequences in the International 1964–1981 nodal congregation were spread along the baseline of the dendogram, and the sequences in the Europe 1972–1991 branch were at the base of the Europe branch. Additions to the International 1964–1981 nodal congregation included sequences from a virus isolated in Russia in 1973 and a virus isolated in New Zealand in 1981; a sequence from a virus from Israel 1978 was added to the International 1961–1986 branch. The Europe 1972–1991 branch (colored gold in Figure 1) contained viruses from Israel (1972, 1975, 1979, and 1988), Italy (1991), and the United Kingdom (1978 and 1986; both used in the previous study [8]). The TCRB vaccine strain (Japan, 1967) sequence segregated onto this branch; in our previous study (8); the TCRB sequence was at the base of the European branch. In summary, while the branching pattern of sequences of viruses isolated internationally before ~1980 (8) was basically preserved, the addition of sequences led to definition of a new branch of viruses from Europe and Israel, isolated between 1972 and 1991.

Newly added sequences of viruses isolated after 1990 segregated primarily onto the three continent-restricted branches defined in our earlier study (8), generally in a geographically consistent manner. These branches, designated Europe, Japan-Hong Kong, and US-Japan in the earlier study (8), are colored green, dark blue, and light blue (the US-Japan branch was redesignated USA 1990–2000; see below). The Europe branch was augmented by sequences of viruses from Italy, Germany, and Israel (1991–1998) and subdivided into two sub-branches, one containing sequences of viruses isolated in Europe from

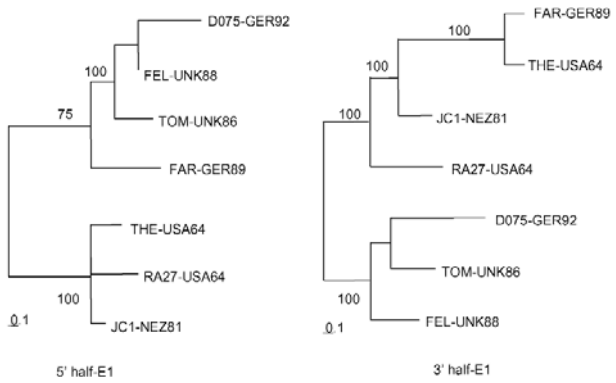


Figure 2. Phylogenetic trees. Trees were made by the maximum likelihood method in the Tree-Puzzle 5.0 program (1,000 puzzling steps) using the 5' half (640 nt: 8291–8930) or 3' half (539 nt: 8931–9469) of the E1 gene sequence. Bootstrapping values (out of 100) for each node are given.

1986 to 1994 (Europe 1986–1994) and a second containing sequences of viruses isolated in Europe and Israel from 1991 to 1995 (Europe 1991–1998). Several newly added sequences of viruses from the United States and Latin America (1997–2000) enlarged the USA 1990–2000 branch; these viruses are thought to be endemic in regions bordering the United States and to seed rubella outbreaks and clusters in that country (10). The only two viruses from Japan in this branch were isolated from a single province in 1991; it was previously hypothesized that this finding represented transport of viruses from the United States to Japan or vice versa (8). In light of the isolation of several viruses in this group subsequently from the United States or Latin America and no viruses from Japan (either before or after 1991), the former transport pathway likely occurred; thus, this branch has been redesignated USA 1990–2000.

The Japan-Hong Kong branch expanded considerably to include sequences of a pair of viruses from Japan and the Philippines (1997) that were related to each other, two sequences of viruses from New Zealand (1991) that were related to each other, and sequences of a group of five related viruses isolated in Japan and South Korea from 1995 to 1996. Another distinct sub-branch was formed by a closely related group of sequences of viruses associated with outbreaks in Europe, the United States, and the Caribbean from 1997 to 2000 (International, 1996–2000). The viruses in this branch caused several outbreaks in the northeastern United States and on cruise ships in Florida (10) and were isolated during a rubella epidemic in Italy in 1997 (9); the relatedness of these viruses was not previously recognized. A novel branch contained sequences of two viruses from China (1999) (China, 1999, gold in Figure 1B).

### RGII Viruses

The tree depicted in Figure 1A indicated that sequence divergence was greater among RGII viruses than among RGI viruses. RGII viruses segregated into three distinct clusters (RGIIA, RGIIB, and RGIIC), all of which were supported by bootstrapping values of >90. Only the RGIIA cluster contained more than one virus isolated within the past decade, and this active cluster contained viruses from diverse locations (India, China, Korea, Italy). The other two clusters contained viruses from a single location (RGIIB-Russia; RGIIC-Beijing, China). The RGII clusters were more distant from each other than were the RGI branches. Variability between RGI and the three RGII clusters is shown in Table 2. Maximal variability among RGI viruses was ~5.8%, however, RGII viruses varied by up to ~8.0%. Viruses within each of the three RGII clusters varied by up to 5.5% and the clusters differed from each other by an average of ~7% (range of variability between viruses in the RGII clusters: RGIIA-RGIIB: 6.35%-7.78%; RGIIA-RGIIC: 6.86%-7.95%; RGIIB-RGIIC: 6.85%-7.53%). Average variation of the RGII clusters from RGI was from ~6% to 8% (range 5.5%-10.3%, which translates to a range of 0.8% to 2.1% variation at the amino acid level).

### Evidence for Recombinant Virus

Use of the entire E1 gene resulted in excessive time being required to run phylogenetic programs with the large number of sequences included in this study (e.g., trees in Figure 1 took days to compute). We therefore investigated using smaller segments within the E1 gene (five windows of ~400 nt encompassing nt 8291–8640, 8491–8890, 8687–9088, 8891–9290, and 9100–9469 of the genome) to construct phylogenetic trees. All major branches shown in Figure 1 were preserved in trees constructed from each of

Table 2. Intra- and intergoup genetic distances among rubella genotype I (RGI) and RGII clusters[a]

| | | Mean distance from | | | |
|---|---|---|---|---|---|
| Genotype/cluster | Intragroup variability | RGII | RGIIA | RGIIB | RGIIC |
| RGI | 0.08–5.75 | 7.28 | 7.59 | 6.20 | 8.21 |
| RGII | 0–7.95 | | | | |
| RGIIA | 0–5.41 | | | 7.24 | 7.13 |
| RGIIB | 0.42–1.95 | | | | 7.19 |
| RGIIC | 2.54 | | | | |

[a]Ranges and mean genetic distances (% nucleotide difference) were determined from all pairwise combinations from viruses in these groups (Figure 1).

these windows, although placement and joining of the branches varied at the base of the tree. During this investigation, the sequence of one isolate, FAR-GER89, was found to group with three contemporaneous viruses in the Europe 1986–1994 branch (TOM-UK86, FEL-UK88, and D075-GER92) in the three 5′-most windows within the E1 gene, but with three International 1964–1981 viruses (RA27-USA64, JC1-NEZ81, and THE-USA64) in the two 3′-most windows (illustrated in Figure 2). When these seven sequences were aligned, over the 5′ 630 nt, 14 nt characteristics of the TOMI/FEL/D075 or THE/JC1/RA27 sequences were identified; of these, FAR had 12 characteristics to the TOMI/FEL/D075 sequences, 1 characteristic to the THE/JC1/RA27, sequences and 1 unique nucleotide (data not shown). In contrast, over the 3′ 540 nt of the E1 gene, 11 nt characteristics of the TOM/FEL/D075 or THE sequence were identified and of these 11 characteristic nts, FAR shared all 11 with the THE sequence and none with the TOM/FEL/D075 sequences. These results indicate that FAR may be a recombinant between viruses from these two Genotype I groups. The FAR-GER89 sequence is on its own branch, emanating from the RGI node (Figure 1); similar observations have been made with poliovirus recombinants (17).

## Discussion

In this study, we extended phylogenetic analysis of rubella viruses collected worldwide. The baseline for this study was an analysis (8) of viruses collected from Europe, North America, and Asia, 1961–1997; we found that an international genotypic group existed until ~1980 and was replaced by continent-restricted genotypic groups after ~1980. In this study, this analysis was augmented by inclusion of comprehensive collections from Italy (9) and the United States (10) as well as viruses collected from new locations. In the previous analysis (8), a second genotype was identified among a limited number of specimens collected from Asia and a specific goal of this study was to analyze viruses from new locations in hopes of learning more about this second genotype.

Use of the entire E1 gene resulted in excessive time being required to run phylogenetic programs with the large number of sequences included in this study. We therefore investigated using ~400-nt segments within the E1 gene. While the major genotypic and subgenotypic grouping were preserved in these segmental trees, the trees produced from the nt 8687–9088 window were most similar to those produced from the entire E1 gene. This window was named the "molecular epidemiology window." As shown in Table 3, this window had a similar to somewhat higher intersequence variability than the E1 gene and preserved the GC content and intersequence transition/transversion ratio exhibited by the E1 gene. Although virus isolates were used in this study, amplification and sequencing of E1 gene segments directly from clinical specimens using reverse transcription-polymerase chain reaction (RT-PCR) are at hand (18–22). Our findings indicate that RT-PCR products from any region of the E1 gene will produce phylogenetic trees consistent with those from the entire E1 gene. However, thus far the regions amplified by in these studies have not been standardized.

Analysis of the segmental trees indicated that the FAR-GER89 isolate might have arisen by a recombination event within the E1 gene between a virus from the Europe 1986–1994 branch (TOM-UK86, FEL-UK88, D075-GER92) and a virus from the International 1964–1981 nodal cluster (THE-US64, RA27-USA64, JC1-NEZ81). Such an event is temporally consistent because viruses related to the International 1964–1981 nodal cluster have been isolated in Italy as late as 1991 (9) but were not included in this study. Although rubella virus recombination in cell culture has been documented (23), this report is the first of a natural recombinant. Although the FAR-GER89 isolate was related to the RA27/3 vaccine strain in the 3′ end of the E1 gene, the FAR-GER89 sequence contains none of the nucleotides characteristic of the RA27/3 sequence (7); thus, the recombination event did not involve a vaccine virus.

In our previous analysis (8), all of the viruses isolated in Europe, North America, and Japan were RGI. This finding was maintained with the expanded collections from all three regions used in this study with the exception of two RGII viruses isolated in Italy. Additionally, most of the viruses in the expanded RGI collection from these regions segued into the previously defined RGI branches (8), with the exception of a newly defined Europe 1972–1991

Table 3. Comparison of genotypic statistics using the E1 gene and the molecular epidemiology window (MEW)

| | | | Intergenotypic distance[a,b] | | | Intragenotypic distances[a,c] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | RGI | | RGII | |
| Window | G+C content[a] | Transition/ transversion[a] | Range | Mean | Mean RGI vs RGII | Range | Mean | Range | Mean |
| E1 | 66.6 | 6.34 | 0.08~10.32 | 4.92 | 7.28 | 0.08~5.75 | 3.55 | 0~7.95 | 5.66 |
| MEW | 66.1 | 6.15 | 0~11.69 | 4.97 | 8.32 | 0~5.97 | 3.49 | 0~8.71 | 6.61 |

[a]Statistics were determined from all sequences listed in Table 1.
[b]The range and mean of genetic distances (% difference) were determined by using all sequences. The mean rubella genotype I (RGI) vs. RGII was determined from all of the pairwise RGI-RGII combinations.
[c]The range and mean of genetic distances were determined for RGI and RGII viruses separately.

branch that contained viruses at the base of the Europe branch defined in the previous study and a novel branch consisting of two viruses from China (1999). In the case of viruses from Europe, with a larger number of viruses representing a longer time span, these viruses belonged to two branches, the second of which divided into two sub-branches. The RGI viruses from Israel were related to the European viruses and fit into the temporal pattern of isolation of the European viruses. The temporal pattern of isolation of viruses in the European branches and sub-branches (1972–1991, 1986–1994, and 1991–1998) indicated that temporal displacement of genotypic groups occurred, as had been noted in our previous study on viruses from Italy (9). These European branches and sub-branches have been more recently displaced by the International 1997–2000 sub-branch of the Japan-Hong Kong branch. The addition of viruses isolated recently in the United States (10) clarified that the US-Japan branch in the previous study (8) was a United States branch, viruses from which, when transported to Japan, caused an outbreak in 1991. Viruses in the USA 1990–2000 branch were related to the limited number of viruses available from Latin America.

The branch termed a Japan-Hong Kong branch in our earlier study (8) contained viruses from Japan and Hong Kong isolated between 1976 and 1991. Ten representative viruses were selected from that branch for this study, including 2 viruses from Japan (1976 and 1977) and 1 virus from Hong Kong (1985) that grouped at the base of the branch as well as several viruses from Japan (1987–1991) that formed a sub-branch. This branch was expanded by the addition of four sub-branches containing viruses from New Zealand, 1991; Japan and Korea, 1994–1996; Japan and the Philippines, 1997; and an international sub-branch that contained viruses associated with epidemics and outbreaks in Europe and the United States during 1997 to 2000 that were unrelated to viruses previously isolated from those regions. Recent evidence indicates that these viruses are closely related to viruses from China (Z. Zhen et al., unpub. data). Thus, the appearance of this sub-branch in Europe and the United States was likely due to intercontinental transport. Considering that viruses from this sub-branch have been predominant in Europe since 1997 and have been one of the two genotypic groups isolated recently in the United States (USA 1990–2000 is the other), an international genotypic branch of rubella virus appears to have emerged after a ~20-year hiatus since the previous demonstrable international branch. Along with this branch, the two other currently active branches are the USA 1990–2000 branch and the China 1999 branch, only represented by two viruses from the collection used in this study.

With its expanded virus collection, this study demonstrates that RGI rubella virus isolates segregate into discrete subgenotypic groups (i.e., branches) that exhibit geographic and temporal consistency. These groups could provide the basis for a standard classification scheme, as has been developed for other viruses. In this study, the number of available RGII viruses also increased to 14 in contrast to the 3 available in the previous analysis; the RGII viruses segregated into three discrete clusters (RGIIA, RGIIB, and RGIIC). RGI viruses formed a discrete cluster clearly distinguishable from the most closely related RGII cluster (RGIIB) with no indication of intermediate viruses. The maximum diversity within RGI was 5.8%, and RGI viruses were an average of >7% different from the RGII viruses (Table 2); when one considers the extensive nature of the RGI virus collection, this is a working definition of a rubella virus genotype. RGII was originally defined to contain a limited number of sequences (three) that distinctly differed from the RGI viruses that made up most of the collection (8). The sequences in each of the RGII clusters vary by up to 5.4%, and the average distance between viruses in each of the clusters is >7% (Table 2). Thus, each of these clusters could represent a genotype. This hypothesis is strengthened by a histogram of the intersequence distances that show a bimodal distribution with peaks from 0.5%-6.0% to 6.5%-11.0%, corresponding to intra- and intergenotypic distances (Figure 3). The bimodal distribu-

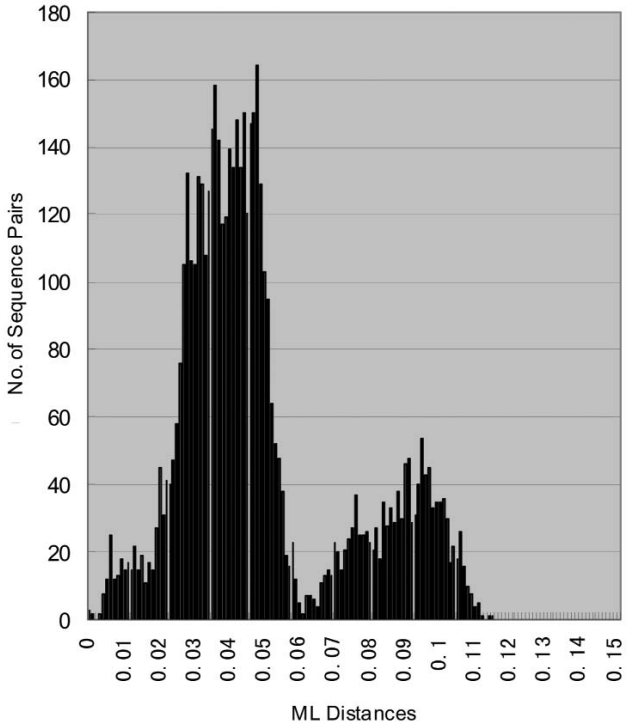


Figure 3. Histogram of genetic distances between rubella virus sequences. The histogram, showing the distribution of all of the pairwise distances between the rubella virus sequences in the study, was constructed from the maximum likelihood distance matrix computed by Tree Puzzle 5.0 program

tion also indicated equivalency of genotypic groups (24). However, in light of the limited number of RGII specimens, more specimens are needed to fully characterize the extent of the diversity within non-RGI viruses, and criteria need to be established for definition of additional genotypes. In this regard, as represented by the viruses in this collection, only one of the RGII clusters (RGIIA) has been repeatedly active in the past decade. However, viruses belonging to the RGII C cluster (China) have been recently isolated (Z. Zhen et al., unpub. data).

Figure 4 shows the countries from which RGI and RGII viruses have been isolated. RGII has not been isolated from an indigenous case outside of the Eastern Hemisphere. Only RGI viruses have been isolated from the United Kingdom, Belgium, Germany, Japan, and New Zealand; other studies have shown RGI viruses in Brazil (10) and Greece (23). Only RGII viruses have been isolated from India, and both genotypes have been isolated from Russia, Italy, Israel, China, and Korea. In Italy and Korea, the two genotypes were isolated in the same year. In both Italy and Israel, isolation of RGII viruses was only during a single year. By contrast, RGI viruses were isolated in both previous and subsequent years. This finding indicates that these RGII viruses were imports, although the relative distant relatedness of the two Italy isolates suggests at least two importation events, albeit in a single year (9). In China, RGII viruses were isolated in 1979 and 1980; the most recent isolates (1999) were RGI, although RGIIC viruses were recently isolated (Z. Zhen et al., unpub. data). These data indicate that RGI has a wider worldwide distribution than does RGII and that in much of the world RGI is the sole genotype. Recent RGII activity is confined to Asia and overlaps with RGI; however, the dataset from areas in which RGII viruses appear to circulate is limited. Additionally, as shown in Figure 4, large regions of the world remain to be sampled to complete the rubella virus genotypic picture.


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Dr. Zheng spent 4 years as a research scientist in the Respiratory and Enteric Viruses Branch (REVB) at the Centers for Disease Control and Prevention (CDC), where he studied the molecular epidemiology of poliovirus, specifically with regard to the elimination of wild poliovirus from China in 1994. He worked as a virologist at the Chinese Center for Disease Control and Prevention in Beijing. Dr. Zheng currently works on Norovirus molecular epidemiology in the REVB at CDC. His scientific research interests are viral molecular genetics and molecular epidemiology.


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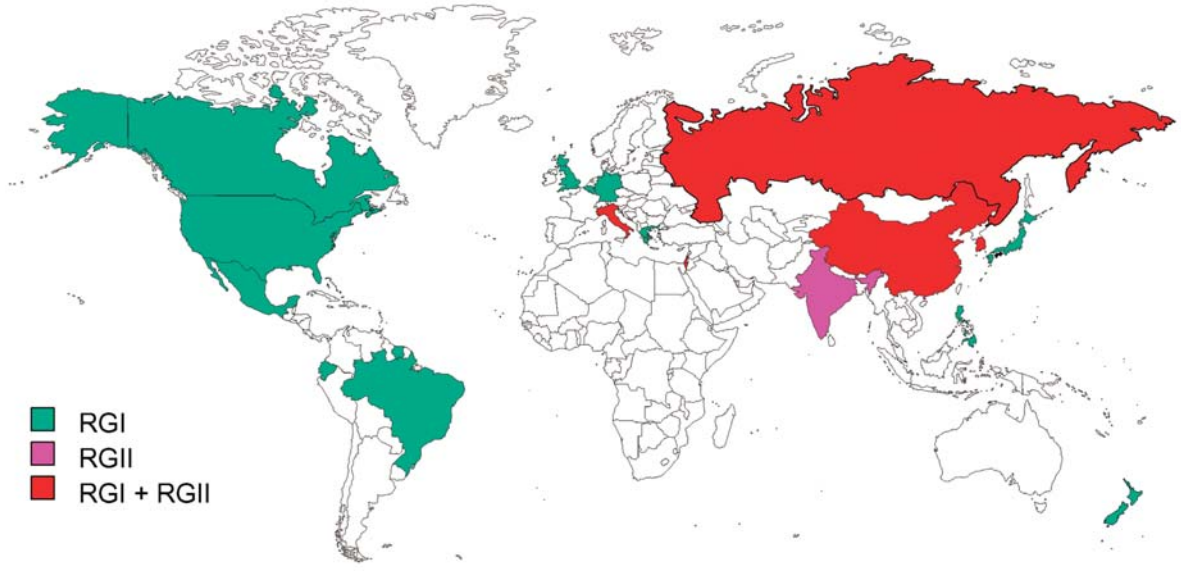


Figure 4. Distribution of rubella genotypes.

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Address for correspondence: Teryl K. Frey, Georgia State University, Biology Department, P.O. Box 4010, Atlanta, GA 30303, USA; fax: 404 651 3105; email: tfrey@gsu.edu

# Risk Factors for Marburg Hemorrhagic Fever, Democratic Republic of the Congo

**Daniel G. Bausch,*[1] Matthias Borchert,†[2] Thomas Grein,‡ Cathy Roth,‡ Robert Swanepoel,§ Modeste L. Libande,¶ Antoine Talarmin,#[3] Eric Bertherat,**[4] Jean-Jacques Muyembe-Tamfum,¶ Ben Tugume,†† Robert Colebunders,† Kader M. Kondé,‡‡[5] Patricia Pirard,§§ Loku L. Olinda,¶ Guénaël R. Rodier,‡ Patricia Campbell,¶¶ Oyewale Tomori,‡‡ Thomas G. Ksiazek,* and Pierre E. Rollin***

We conducted two antibody surveys to assess risk factors for Marburg hemorrhagic fever in an area of confirmed Marburg virus transmission in the Democratic Republic of the Congo. Questionnaires were administered and serum samples tested for Marburg-specific antibodies by enzyme-linked immunosorbent assay. Fifteen (2%) of 912 participants in a general village cross-sectional antibody survey were positive for Marburg immunoglobulin G antibody. Thirteen (87%) of these 15 were men who worked in the local gold mines. Working as a miner (odds ratio [OR] 13.9, 95% confidence interval [CI] 3.1 to 62.1) and receiving injections (OR 7.4, 95% CI 1.6 to 33.2) were associated with a positive antibody result. All 103 participants in a targeted antibody survey of healthcare workers were antibody negative. Primary transmission of Marburg virus to humans likely occurred via exposure to a still unidentified reservoir in the local mines. Secondary transmission appears to be less common with Marburg virus than with Ebola virus, the other known filovirus.

Marburg hemorrhagic fever (MHF) is a severe illness caused by Marburg virus, a member of the *Filoviridae* family. MHF was first described in 1967 during outbreaks in Germany and the former Yugoslavia that were linked to monkeys imported from Uganda (1–3). Since then, only a few sporadic cases in East Africa and southern Africa and one laboratory infection have been identified (4–7). Serosurveys for Marburg antibodies in the general population generally have shown prevalences of <2%, indicating it to be a rare and highly lethal disease (8–25).

The largest outbreak of MHF recorded to date began in late 1998 in northeastern Democratic Republic of the Congo (DRC) (26,27). Although the remoteness of the area and the civil war in eastern DRC delayed access and evaluation, in May 1999 a team of international investigators identified 73 cases (8 laboratory-confirmed and 65 suspected cases retrospectively identified) (28). Follow-up surveillance subsequently identified >150 cases through December 2000.

The natural reservoir for Marburg virus remains unknown, although it is presumed to be of zoonotic origin. Primary transmission of the virus from the natural reservoir appears to occur only in sub-Saharan Africa and is sometimes followed by secondary person-to-person transmission in both community and nosocomial settings (4–6,29). Because of the disease's rarity and lethality, risk factors for transmission of Marburg virus have not been extensively investigated. We therefore performed two antibody surveys in the wake of the 1998–99 outbreak in DRC to explore risk factors for Marburg virus exposure and transmission. One antibody survey was a cross-sectional study of the general village populations; the other was a focused investigation of healthcare workers (HCWs).

*Centers for Disease Control and Prevention, Atlanta Georgia, USA; †Institute of Tropical Medicine, Antwerp, Belgium; ‡World Health Organization, Geneva, Switzerland; §National Institute for Communicable Diseases, Johannesburg, South Africa; ¶Ministry of Health, Kinshasa, Democratic Republic of the Congo; #Institut Pasteur, Cayenne, French Guiana; **Le Pharo, Marseille, France; ††Uganda Virus Research Institute, Entebbe, Uganda; ‡‡World Health Organization, AFRO, Harare, Zimbabwe; §§Doctors without Borders, Brussels, Belgium; and ¶¶Doctors without Borders, Amsterdam, the Netherlands

[1]Present address: Tulane School of Public Health and Tropical Medicine, New Orleans, LA, USA.
[2]Present address: London School of Hygiene and Tropical Medicine, London, England.
[3]Present address: Institut Pasteur, Bangui, Central African Republic.
[4]Present address: World Health Organization, Geneva, Switzerland.
[5]Present address: World Health Organization, AFRO, Ouagadougou, Burkina Faso.

## Methods

### Area of Study

The studies we describe were performed as an adjunct to the investigation of an outbreak of MHF in May 1999. The epicenter of the outbreak was the village of Durba in the Haut-Uélé District, Oriental Province, in northeastern DRC, an isolated region approximately 200 km from the borders of Uganda and Sudan (Figure). Although no official population count for Durba is available, unofficial estimates are approximately 25,000. Watsa, a larger town of approximately 60,000 and the administrative seat of the zone, lies 14 km away. Although the Yogo ethnic group predominates, the population of Durba/Watsa is quite heterogeneous, as many people have migrated to the area to work in the local gold mines. Most are Catholic. The area has had intermittent armed conflict since the beginning of the Congolese civil war in 1996, a situation that has severely limited travel and economic growth.

The livelihood of most of the population in the Durba/Watsa area is associated with gold mining, conducted almost exclusively by young men and most often without professional training or equipment. Some older men, women, and children are involved in the extraction of gold from ore and its sale. Subsistence farming and hunting are also common. Although various mines exist in the area, most mining appears to take place in the Goroumbwa mine a few kilometers from the village of Durba. In addition, some miners dive in local rivers in search of gold. The existence of a hemorrhagic illness in the region appeared to be common knowledge and was labeled "Durba hemorrhagic syndrome" or "Durba syndrome" by the villagers, who often associated it with working in the mines.

Because of the remoteness of the region and the war, supplies are severely limited in all the health facilities in the region. The major facility in Durba is a small rudimentary government health center staffed by a few nurses. In Watsa, there is a larger government hospital, a hospital affiliated with the mining company, and two government health centers. In addition, at least 14 small private health centers operate collectively in Durba and Watsa.

### Study Population

Two surveys were undertaken. The first was a cross-sectional survey on a convenience sample of the general population of Durba township. It was performed by establishing a post in the center of the village. With the aid of local HCWs and a village loud-speaker system, residents of sequential "quartiers" of the village were requested to come for evaluation over a 3-day period. Persons <15 years of age were excluded. The second survey focused on HCWs at all health centers in Durba and Watsa. All HCWs were surveyed at their place of employment.


Figure. Map of the Democratic Republic of the Congo indicating the neighboring villages of Durba and Watsa, the epicenter of the 1998–1999 outbreak of Marburg hemorrhagic fever.

### Questionnaire

The rationale for conducting the study was explained to all participants, and verbal consent was obtained. Questionnaires were pretested on local villagers not included in the final study. For the general population, a 2-page questionnaire was administered. Supervisors at the local mining company were consulted about appropriate questions regarding exposures in the mines. Persons were first evaluated by local village HCWs, who determined their ability to speak and understand French. If deemed able to do so, the person was then interviewed by either a local HCW or a French-speaking member of the international investigative team. For those persons who did not understand French, the questionnaire was administered by a local HCW in the appropriate local language. Participants were asked about their entry into and activities within the mines, exposures to persons presumably sick with Durba syndrome (defined as a severe illness with high fever and bleeding from the nose, mouth, and/or anus) in the hospital and at home, and exposures to various animals thought to possibly transmit Marburg virus (rodents, bats, and monkeys). Participants were given a small bag of peanuts as a token of appreciation for their cooperation.

For the study of HCWs, a 1-page questionnaire was administered; HCWs were asked about exposure to persons with suspected Durba syndrome at work and at home, as well as any history of a compatible illness in the HCWs themselves. HCWs were also asked if they had ever entered the mines. Interviews were conducted in French.

### Phlebotomy and Serologic Testing

After administration of the questionnaire, 5 mL of blood was obtained and stored out of the sunlight. At the

end of the day, the serum and clot were separated, labeled, and stored in liquid nitrogen. Aliquots were sent on dry ice for analysis at both the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA, and the National Institute for Communicable Diseases in Johannesburg, South Africa.

Testing for Marburg-specific immunoglobulin (Ig) M and IgG antibodies by the enzyme-linked immunosorbent assay (ELISA) was conducted with a technique analogous to that reported for detecting antibody to Ebola virus, except for the substitution of an antigen made from the Musoke strain of Marburg virus (30,31). The cut-off value for a positive ELISA result was set to 3 standard deviations from the mean control-adjusted optical density (OD) of 410 nm found on a panel of normal serum samples. This value generally corresponds to an OD of approximately 0.1 at a dilution of 1:100 for the IgM assay, and 0.2 at 1:400 for the IgG assay, which generally has a higher background. Positive and negative controls were included with each run and consisted of serum from African patients with and without a laboratory-confirmed history of MHF. Because the rarity of MHF has precluded rigorous field testing of the ELISA for Marburg antibody, all ELISA-positive serum samples were also examined by the immunofluorescent antibody assay (IFA), as previously described (32). The cut-off titer for a positive IFA result was 1:50. Serum samples were considered positive only if positive results were obtained by both ELISA and IFA. Study participants were directed to seek the serologic results (free of charge) 1 month after testing from the nurse in charge of the local health center. The nurse was provided the results along with details on how to interpret antibody status.

### Data Analysis

Questionnaires were created by using EpiInfo version 6.04 (CDC, Atlanta, GA). Data were initially recorded on the questionnaires by hand, then entered into EpiInfo 6.04, and finally imported into SPSS version 10.0 statistical software (SPSS, Chicago, IL) for further analysis. The chi-square test, Fisher exact test, and Student t test with Levene's test for equality of variances were employed, where appropriate. Data found to be non-normally distributed were normalized by a log 10 transformation before statistical analysis. Statistical tests were two-sided, and significance was set at $p \leq 0.05$. Variables with significance values of $p \leq 0.1$ in the univariate analysis were examined in a multivariate model using forward stepwise maximum likelihood logistic regression.

## Results

### General Population

A total of 912 participants were surveyed. Another seven persons were initially enrolled but did not stay to complete their questionnaires or to have a blood sample drawn. No further information is available regarding these seven or their reasons for withdrawal. Demographic data are presented in Table 1. Since the gold deposits in Durba attract young male miners from the surrounding region, most participants were men (65%), and 347 (38%) listed their occupation as miner, although only 281 (81%) of these 347 were currently working in the mines. Virtually all (99%) of the miners were male. Marburg-specific IgG antibodies were found in 15 (2%) of the 912 study participants. All were IgM antibody–negative. Thirteen (87%) of the 15 IgG antibody–positive participants were male miners. The other two were women who had never entered into the mines and whose profession was listed as "other" (most likely housewives).

All 13 of the antibody-positive miners were currently working: 10 (77%) at Goroumbwa, 2 (15%) at another mine in Durba, and 1 (8%) at an unspecified site. None reported river-diving for gold. Compared with those who were antibody-negative, antibody-positive miners tended to have worked fewer years at their present mine site but to have spent more time in the mines, although the differences were not statistically significant (Table 2). Antibody-positive miners were significantly younger than their antibody-negative counterparts (Table 1). This finding perhaps reflects the longer exposure time in the mines of younger miners (age <30 years) relative to older miners (mean $\pm$ standard error of the mean [SEM] consecutive hours per week 17.7 $\pm$ 1.2 vs. 13.7 $\pm$ 1.3, respectively, p = 0.006;

Table 1. Demographic characteristics and Marburg immunoglobulin G antibody results of the study population in Durba, Democratic Republic of the Congo, 1999[a]

| Characteristic | All participants n = 912 (%) | IgG antibody positive n = 15 (%) | IgG antibody negative n = 897 (%) | OR (95% CI) | p value |
|---|---|---|---|---|---|
| Male | 594 (65) | 13 (87) | 581 (65) | 3.5 (0.8 to 15.4) | 0.10 |
| Mean age, y (range) | 31 (14–79) | 27 (21–42) | 31 (14–79) | - | 0.04 |
| Profession | | | | - | |
| Miner | 347 (38) | 13 (87) | 334 (37) | 11.0 (2.5 to 48.9) | < 0.001 |
| Merchant | 141 (15) | 0 (-) | 141 (16) | - | 0.15 |
| Other/unknown | 424 (46) | 2 (13) | 422 (47) | - | - |

[a]Odds ratios (OR) and p values are for the comparison between antibody-positive and -negative participants. CI, confidence interval; Ig, immunoglobulin.

Table 2. Duration of time spent working in mines and Marburg immunoglobulin G antibody status among 281 active miners in Durba, Democratic Republic of the Congo, 1999

| Time in mines | Antibody positive (± SEM) (n = 13) | Antibody negative (± SEM) (n = 268) | p value |
|---|---|---|---|
| At present mine site (y) | 6.6 ± 1.0 | 10.3 ± 0.6 | 0.52 |
| Usual h/wk working in mine | 58.2 ± 9.2 | 49.5 ± 1.7 | 0.36 |
| Usual h in mine without exiting | 24.2 ± 6.1 | 16.0 ± 0.9 | 0.07 |
| Longest stint in mine (h) | 38.8 ± 10.2 | 28.8 ± 1.8 | 0.16 |

mean ± SEM longest stint: 34.7 ± 2.3 vs. 23.5 ± 2.1, respectively, $p < 0.001$).

We examined associations between antibody to Marburg virus and various practices while working in the mines, as well as exposures related to sick persons in the home, healthcare services, and animals. In a univariate analysis, significant positive associations were found with having touched the corpse of someone who died from Durba syndrome, having had Durba syndrome oneself, and having received injections in the past year (Table 3). Touching the blood, feces, or urine of someone with Durba syndrome was of borderline statistical significance. Consumption of rodents was associated with a borderline significant protective effect, which was probably a spurious association. Of the four antibody-positive survey participants who said they had had Durba syndrome themselves, three dated the illness to the 5 months immediately before the study. The fourth, although uncertain, dated his illness to September 1998, 9 months before the investigation.

Receiving an injection as part of medical treatment was common in Durba: 505 (56%) of 907 of the participants in our cross-sectional village survey reported receiving an injection in the previous year, including 13 (87%) of the 15 antibody-positive participants (11 miners and both female nonminers). Overall, however, receiving injections was significantly more common among nonminers than miners (62 [368/596] vs. 47 [162/348], respectively, OR 1.9, 95% CI 1.4 to 2.4, $p < 0.001$) and among women than men (71% [221/312] vs. 49% [305/626], respectively, OR 2.6, 95% CI 1.9 to 3.4, $p < 0.001$).

In a multivariate model, the only variables that remained significantly associated with a positive Marburg antibody result were being a miner (OR 13.8, 95% CI 3.1 to 62.1) and having received injections (OR 7.4, 95% CI 1.6 to 33.2). Having previously had Durba syndrome was not added to the model, as it was not an independent risk factor for acquiring MHF. The associations between Marburg antibody, mining, and receiving injections remained essentially unaltered when men were looked at independently. The number of antibody-positive women (two) was too small to permit meaningful statistical analysis. However, both positive women were among the relatively few survey participants with extensive secondary contact in the household. Both reported having someone in the household sick with Durba syndrome, having contact with their body fluids, and participating in their burial, although only one of the two women reported direct contact with the corpse. In contrast, 4 (31%) of the 13 antibody-positive male miners reported any type of household exposure.

### Healthcare Workers

One hundred three HCWs were enrolled from 15 different health centers, including 73 (71%) nurses, 13 (13%) clerical or administrative staff, 10 (10%) midwives, 5 (5%) laboratory workers, and 2 (2%) doctors. These figures are thought to represent virtually all of the active HCWs in the two villages except those practicing traditional medicine. HCWs had a mean of 9 years (range 0–42) of experience. All were negative for both Marburg IgM and IgG antibodies, despite the fact that 67 (65%) reported caring for a patient with Durba syndrome, and 5 (5%) reported having had Durba syndrome themselves. Types of patient contact included administering injections (38%); cleaning up blood, vomitus, urine, or feces (28%); washing bed clothes (7%); washing corpses (6%); and receiving a needlestick injury (2%).

## Discussion

Despite conclusive evidence of circulation of Marburg virus in the Durba/Watsa area in the months and years preceding our antibody surveys, we found few persons with serologic evidence of previous infection (26,27). This likely reflects a combination of the rarity of MHF and the high case-fatality ratio (83%) associated with the disease in Durba/Watsa, leaving few survivors for study.

Most previous observations on risk factors for MHF have been of an anecdotal nature. Despite the small number of antibody-positive survey participants found in Durba, we were able to systematically identify and quantify several risk factors for MHF. The preponderance of antibody in male miners without obvious evidence for person-to-person transmission suggests that the local mines are a site of primary infection with Marburg virus, most likely through exposure to the primary zoonotic reservoir. Various previous findings support the conclusion of an association between MHF and exposure in mines and caves, including the following: 1) most cases of MHF identified in Durba/Watsa through December 2000 occurred in miners (J.J. Muyembe-Tamfum et al., unpub. data); 2) molecular epidemiologic data demonstrate the

Table 3. Antibody to Marburg virus and possible risk factors for Marburg hemorrhagic fever in Durba, Democratic Republic of the Congo, 1999[a]

| Characteristic | All participants (%) | Antibody positive (%) | Antibody negative (%) | OR (95% CI) | p value |
|---|---|---|---|---|---|
| Behavior in the mines[b] | | | | | |
| Wear mask | 4/289 (1) | 1/13 (8) | 3/276 (1) | 7.6 (0. to 78.4) | 0.17 |
| Drink water from sources in the mine | 160/289 (55) | 9/13 (69) | 151/276 (55) | 1.9 (0.6 to 6.2) | 0.40 |
| Use explosives | 129/289 (45) | 7/13 (54) | 122/276 (44) | 1.5 (0.5 to 4.5) | 0.57 |
| Wear boots | 46/289 (16) | 2/13 (15) | 44/276 (16) | 1.0 (0.2 to 4.5) | 1.00 |
| Household/village exposures to someone with Durba syndrome[c] | | | | | |
| Touched corpse | 88/905 (10) | 4/15 (27) | 84/890 (9) | 3.5 (1.1 to 11.2) | 0.05 |
| Touched blood, feces, or urine | 60/903 (7) | 3/15 (20) | 57/888 (6) | 3.6 (1.0 to 13.3) | 0.07 |
| Worked with someone with syndrome | 248/906 (27) | 7/15 (47) | 241/891 (27) | 2.4 (0.8 to 6.6) | 0.15 |
| Been in the same room with someone with syndrome | 179/902 (20) | 4/15 (27) | 175/887 (20) | 1.5 (0.5 to 4.7) | 0.51 |
| Touched skin of person during illness | 286/903 (32) | 6/15 (40) | 280/888 (32) | 1.4 (0.5 to 4.1) | 0.58 |
| Someone in the household sick with syndrome | 210/906 (23) | 4/15 (27) | 206/891 (23) | 1.2 (0.4 to 3.8) | 0.76 |
| Participated in burial | 393/904 (43) | 6/15 (40) | 387/889 (44) | 0.9 (0.3 to 2.5) | 1.00 |
| Healthcare-related exposures | | | | | |
| Had Durba syndrome yourself | 60/912 (7) | 4/15 (27) | 56/897 (6) | 5.4 (1.7 to 17.7) | 0.01 |
| Received injections in the last year | 505/907 (56) | 13/15 (87) | 492/892 (55) | 5.2 (1.2 to 23.6) | 0.02 |
| Underwent surgery in the last year | 85/905 (9) | 2/15 (13) | 83/890 (9) | 1.5 (0.3 to 6.7) | 0.64 |
| Received scarification[d] in the last year | 209/906 (23) | 4/15 (27) | 205/891 (23) | 1.2 (0.4 to 3.9) | 0.76 |
| Animal exposures | | | | | |
| Rodents | | | | | |
| Touched | 437/897 (49) | 4/14 (29) | 433/883 (49) | 0.4 (0.1 to 1.3) | 0.18 |
| Ate | 271/892 (30) | 1/15 (7) | 270/877 (31) | 0.2 (0.0 to 1.2) | 0.05 |
| Bitten by | 200/896 (22) | 3/15 (20) | 197/881 (22) | 0.9 (0.2 to 3.1) | 1.00 |
| Bats | | | | | |
| Touched | 169/901 (19) | 4/14 (29) | 165/887 (19) | 1.8 (0.5 to 5.6) | 0.31 |
| Ate | 31/898 (3) | 0/15 (-) | 31/883 (4) | - | 1.00 |
| Bitten by | 8/896 (1) | 0/15 (-) | 8/881 (1) | - | 1.00 |
| Monkeys | | | | | |
| Touched | 502/892 (56) | 6/14 (43) | 496/878 (57) | 0.6 (0.2 to 1.7) | 0.42 |
| Ate[e] | 682/895 (76) | 11/14 (79) | 671/881 (76) | 1.1 (0.3 to 4.2) | 1.00 |
| Bitten by | 76/895 (8) | 1/15 (7) | 75/880 (9) | 0.8 (0.1 to 5.9) | 1.00 |

[a]Odds ratios (OR) and p values are for the comparison between antibody-positive and -negative participants. CI, confidence interval.
[b]Includes only responses from persons who stated that they currently worked in the mines.
[c]Before questioning began, Durba syndrome was described to the participant as "a severe illness with high fever and bleeding from the nose, mouth, and/or anus."
[d]Scarification is the practice of intentionally scarring the skin with sharp instruments. It may be done for aesthetic reasons or because of a belief that it has medicinal or spiritual value.
[e]Many participants reported the meat was smoked or cured at the time of purchase, so potential exposure to viable virus may have been limited.

circulation of numerous distinct genotypes of Marburg virus in Durba/Watsa, consistent with multiple parallel primary introductions rather than a single one amplified by secondary spread (R. Swanepoel et al., unpub. data); and 3) previous cases of MHF have been associated with entry into caves (5,6).

As expected, close contact with case-patients with MHF or corpses were risk factors for secondary transmission of Marburg virus. Although injection with contaminated syringes has been previously shown to be associated with filovirus transmission, the retrospective nature of our study makes it impossible to discern whether the use of Marburg virus-contaminated syringes resulted in virus transmission in Durba/Watsa or whether patients sick with MHF, usually a severe disease, were simply more likely to seek and receive medical care, including injections (33,34). That the general profile of the antibody-positive persons who received injections (male miners) contrasted with that of the general population (female, nonminer) suggests that the latter explanation may be more likely.

Although at least seven HCW infections have been confirmed in Durba/Watsa since 1998, we found no antibody-positive HCWs, despite what would appear to be frequent high-risk exposures (35, J.J. Muyembe-Tamfum et al., unpub. data). The high case-fatality ratio may again explain the absence of antibody-positive HCWs, although historical review does not suggest the existence of previous large nosocomial epidemics in Durba/Watsa (D. Bausch et al., unpub. data). Sound barrier nursing practices on behalf of local HCWs may have helped prevent nosocomial transmission but, given the severely limited availability of protective material in the area, this is unlikely to be the sole explanation.

The low prevalence of Marburg antibody found in Durba/Watsa, despite what would be considered significant risk factors for person-to-person transmission, sug-

gests that secondary transmission of Marburg virus may be relatively infrequent compared with transmission of the other known member of the filovirus family, Ebola virus. In contrast to Ebola hemorrhagic fever (33,34), no large nosocomially amplified outbreaks of MHF have been noted. Only six secondary infections (five nosocomial and one sexually transmitted) were noted of the 32 cases reported during the original MHF outbreak in 1967 in Europe, despite the fact that the etiologic agent was unknown at the time of the outbreak and thus appropriate barrier nursing measures were unlikely to have been rapidly implemented (1–3,36–38). Smith et al. reported that 1 of 207 close contacts of a case-patient with MHF contracted the virus (5). Neither MHF nor antibody developed in a nurse in Durba who suffered a needlestick injury while caring for a case-patient with laboratory-confirmed and subsequently fatal MHF during the 1999 outbreak in DRC; however, the needle and IV line may have been flushed before the accident. Finally, immunohistochemical studies of skin biopsy specimens from patients with fatal MHF generally show that Marburg virus antigen is more sparsely distributed relative to Ebola antigen in fatal cases of Ebola hemorrhagic fever, which suggests that there may be less cutaneous shedding of Marburg virus and thus lower person-to-person communicability in MHF (S. Zaki et al., pers. commun.).

Our study had several limitations. As participants were not randomly selected, disproportionate participation from specific subpopulations could have skewed our results. Selective participation could have occurred because of fear of stigmatization or selective migrations of persons into or out of Durba/Watsa. Social stigmas could have also resulted in recall bias. The small number of Marburg antibody–positive participants limits our statistical power to identify all possible risk factors for MHF. Although ELISA testing for Marburg antibody has not undergone rigorous field testing, we believe that our conservative criterion of positive results on both ELISA and IFA for a participant to be considered Marburg antibody–positive lends credence to our conclusions. The precise duration of antibody persistence after Marburg infection is unknown for both tests. If reversion to antibody-negative status appears after a relatively short time, some previously infected persons may have escaped detection. However, most of the aforementioned limitations would likely result in false-negative results, with the ultimate effect of underestimating the magnitude of any recognized associations.

In defining risk factors for primary transmission of Marburg virus in Durba/Watsa, our study helps orient the hunt for the reservoir for the filoviruses. If primary infection to humans is indeed occurring in the mines around Durba/Watsa, future investigations of the reservoir for Marburg virus should focus on fauna present in such habitats. Bats, rodents, arthropods, and plant life within cave/mine habitats would be the prime suspects. Samples taken from small mammals captured in and around mines in Durba are being analyzed for possible Marburg virus infection (R. Swanepoel, pers. commun.). Only a combination of the use of epidemiologic and epizootiologic investigations along with direct observations made during outbreaks is likely to shed light on the still-cryptic natural history of the filoviruses.

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Dr. Bausch is a medical epidemiologist specializing in the investigation and control of viral hemorrhagic fevers.

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Address for correspondence: Daniel G. Bausch, Tulane School of Public Health and Tropical Medicine, Department of Tropical Medicine, SL-17, J. B. Johnston Building, Rm. 511, 11430 Tulane Avenue, New Orleans, LA 70112-2699 USA; fax: 504-988-6686; email: dbausch@Tulane.edu

# Intensity of Rainfall and Severity of Melioidosis, Australia

**Bart J. Currie* and Susan P. Jacups***

In a 12-year prospective study of 318 culture-confirmed cases of melioidosis from the Top End of the Northern Territory of Australia, rainfall data for individual patient locations were correlated with patient risk factors, clinical parameters, and outcomes. Median rainfall in the 14 days before admission was highest (211 mm) for those dying with melioidosis, in comparison to 110 mm for those surviving (p = 0.0002). Median 14-day rainfall was also significantly higher for those with pneumonia. On univariate analysis, a prior 14-day rainfall of ≥125 mm was significantly correlated with pneumonia (odds ratio [OR] 1.70 [confidence interval [CI] 1.09 to 2.65]), bacteremia (OR 1.93 [CI 1.24 to 3.02]), septic shock (OR 1.94 [CI 1.14 to 3.29]), and death (OR 2.50 [CI 1.36 to 4.57]). On multivariate analysis, rainfall in the 14 days before admission was an independent risk factor for pneumonia (p = 0.023), bacteremic pneumonia (p = 0.001), septic shock (p = 0.005), and death (p < 0.0001). Heavy monsoonal rains and winds may cause a shift towards inhalation of *Burkholderia pseudomallei*.

Melioidosis, infection with *Burkholderia pseudomallei,* is endemic in Southeast Asia and northern Australia (1). Within the disease-endemic region, reported incidence has been increasing; melioidosis is now recognized as the most common cause of severe community-acquired sepsis in parts of northeast Thailand (2) and the most common cause of fatal community-acquired bacteremic pneumonia in the tropical "Top End" of the Northern Territory of Australia (3). The recognized endemic region for melioidosis has also been expanding, with recent reports from Taiwan (4), China, and India (1). Sporadic foci of melioidosis have occurred in temperate locations, possibly resulting from introduced infection (1,5). Melioidosis is also an important infection to consider in travelers returning from a disease-endemic region (6,7). While most cases are from recent infection with *B. pseudomallei*, latency is well recognized, and disease has occurred up to 29 years after a person has left a melioidosis-endemic area (8).

*Menzies School of Health Research and Flinders University, Darwin Northern Territory, Australia

The association between rainfall and melioidosis has long been recognized, with 75% and 85% of cases occurring in the wet season in northeast Thailand (9) and northern Australia (3), respectively. In both regions, the number of seasonal cases correlates with total rainfall.

*B. pseudomallei* is an environmental bacterium of soil and surface water in disease-endemic locations. We have previously documented the incubation period for melioidosis from defined inoculating events to be 1–21 (mean 9) days (10). While most cases are considered to be from percutaneous inoculation (10,11), inhalation is also well recognized as a mode of infection. We have noted that melioidosis patients are often more severely ill after heavy monsoonal rainfall. We now show that intensity of rainfall is an independent predictor of melioidosis in persons admitted to hospital with pneumonia and of death. We postulate that heavy rainfall results in a shift towards inhalation as the mode of infection with B. pseudomallei, which leads to more severe illness.

## Methods

### Patients

The Darwin prospective melioidosis study has documented 318 culture-confirmed cases of melioidosis that occurred in the Top End of the Northern Territory in the 12 years from October 1989 until October 2001. Patient data are stored using Oracle software, version 8.0.4 (Oracle, North Sydney, Australia). Patient variables, as defined previously (3), include age, sex, ethnicity (aboriginal, non-aboriginal), location, and risk factors, including diabetes, alcohol excess, chronic lung disease, smoking, chronic renal disease, and kava use. Clinical parameters include nature of primary melioidosis signs and symptoms (pneumonia, other), presence of bacteremia, septic shock (presence of hypotension not responsive to fluid replacement together with hypoperfusion abnormalities manifest as end organ dysfunction) (12), and outcome (death, survival).

### Rainfall Data

The Top End covers 516,945 km². Daily rainfall data from 12 recording stations, located throughout the region and including major remote aboriginal communities, were provided by the Bureau of Meteorology in Darwin. From these data we calculated the rainfall at each patient's location for defined periods before date of admission. Given a mean incubation period of 9 days for acute melioidosis, we used rainfall in the 14 days before admission for each patient (14-day rainfall) to broadly reflect the rainfall exposure around the infecting event.

### Statistical Analysis

Statistical analyses were performed by using Intercooled STATA 7.0 (Stata, College Station, TX). Initially, median 14-day rainfall was compared for patient variables and clinical parameters. Analysis by t tests was performed after the rainfall data were normalized by using square root transformation. Subsequently, univariate and multivariate analysis was performed with the outcomes being the various clinical parameters. Categorical variables included were age (<45 years, ≥45 years), sex, ethnicity, diabetes, alcohol excess, chronic lung disease, smoking, chronic renal disease, kava use, absence of any risk factors (those listed above or age ≥45 years or cardiac failure, malignancy, or immunosuppressive therapy) and 14-day rainfall (<125 mm, ≥125 mm). Separate multivariate analysis was also performed with normalized 14-day rainfall data as a continuous variable. All logistic regressions were performed by using stepwise forwards technique to find the most parsimonious and significant model.

## Results

The Figure shows the close association between total monthly rainfall, as recorded at Darwin Airport, and the number of cases of melioidosis in the Top End for each month during the 12 years. The correlation between monthly cases of melioidosis and rainfall at Darwin Airport in the preceding calendar month ($r = 0.617$; $p < 0.0001$) was slightly tighter than the correlation with rainfall in the concurrent month ($r = 0.574$).

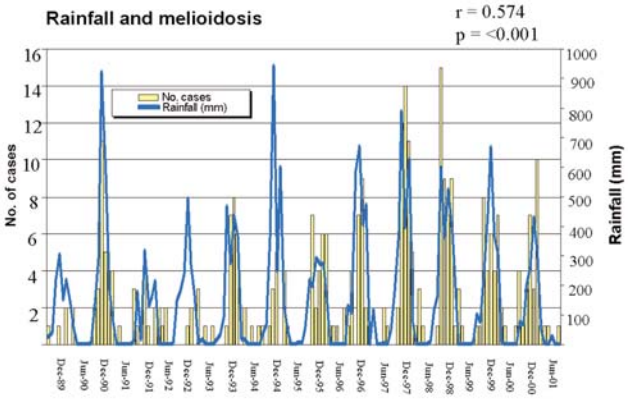


Figure. Monthly rainfall and melioidosis cases during 12- year study period, Australia.

Table 1 shows the median 14-day rainfall correlation with various risk factors, clinical signs and symptoms, and outcomes. The correlation with median 14-day rainfall was significantly higher for patients with pneumonia, those with bacteremia and septic shock, and those who died. For those patients with pneumonia, correlation with median 14-day rainfall was significantly higher if they were bacteremic. Patients with diabetes, alcohol excess, and chronic renal disease were all associated with significantly higher median 14-day rainfall; correlation with 14-day rainfall did not significantly differ for age, sex, ethnicity, chronic lung disease, and smoking (data not shown).

Table 2 shows that, on univariate analysis, 14-day rainfall ≥125 mm correlated significantly with primary symptoms of pneumonia, bacteremia, and septic shock, and with death. Table 3 shows independent predictors of clinical signs and symptoms and outcome when multivariate analysis with 14-day rainfall as a categorical variable was used. When 14-day rainfall was used as a continuous variable, it was an independent risk factor for admission with primary symptoms and signs of pneumonia ($p = 0.023$), bacteremic pneumonia ($p = 0.001$), septic shock ($p = 0.005$), and of death ($p < 0.0001$). Notably, while absence of any risk factors was a predictor of primary signs and symptoms other than pneumonia and of less severe disease, neither

Table 1. Prior 14-day rainfall correlations with risk factors, clinical signs and symptoms, and outcomes

| | Yes | | No | | |
|---|---|---|---|---|---|
| Parameter | No. | Median 14-d rainfall (mm) | No. | Median 14-d rainfall (mm) | p value |
| Diabetes | 119 | 174 | 199 | 102 | 0.002 |
| Alcohol excess | 118 | 132 | 200 | 116 | 0.043 |
| Chronic renal disease | 27 | 198 | 291 | 113 | 0.048 |
| Pneumonia | 156 | 161 | 162 | 105 | 0.001 |
| Bacteremia | 155 | 166 | 163 | 97 | <0.0001 |
| Pneumonia–bacteremic | 86 | 188 | 70 | 120 | 0.035 |
| Nonpneumonia–bacteremic | 71 | 136 | 91 | 89 | 0.007 |
| Septic shock | 74 | 191 | 244 | 112 | 0.0008 |
| Death | 56 | 211 | 262 | 110 | 0.0002 |

Table 2. Univariate analysis for correlation with prior 14-day rainfall of ≥125 mm

| Clinical parameter | | 14-d rainfall ≥125 mm | 14-d rainfall <125 mm | Odds ratio (95% CI)[a] | p value |
|---|---|---|---|---|---|
| Pneumonia | Yes | 88 | 68 | 1.70 (1.09 to 2.65) | 0.019 |
| | No | 70 | 92 | | |
| Bacteremia | Yes | 90 | 65 | 1.93 (1.24 to 3.02) | 0.004 |
| | No | 68 | 95 | | |
| Bacteremic pneumonia | Yes | 53 | 33 | 1.94 (1.17 to 3.21) | 0.010 |
| | No | 105 | 127 | | |
| Septic shock | Yes | 46 | 28 | 1.94 (1.14 to 3.29) | 0.014 |
| | No | 112 | 132 | | |
| Death | Yes | 38 | 18 | 2.50 (1.36 to 4.57) | 0.003 |
| | No | 120 | 142 | | |

[a]CI, confidence interval.

diabetes, alcohol excess, nor chronic renal disease was an independent predictor of signs and symptoms, disease severity, or death.

## Discussion

Our data confirm our observations that patients admitted with melioidosis 1–2 weeks after heavy monsoonal rainfall are more ill and more likely to die. Median rainfall in the 14 days before admission was highest (211 mm) for those who died with melioidosis. For those admitted with bacteremic pneumonia, prior 14-day median rainfall was 188 mm, compared with 89 mm in patients who were nonbacteremic and did not have pneumonia. Multivariate analysis showed that rainfall in the 14 days before admission was an independent predictor of septic shock and death. Patients were 2.5 times more likely to die from melioidosis if the rainfall in the 14 days before admission was ≥125 mm. Overall, 68% of deaths occurred in this high rainfall group. Furthermore, prior heavy rainfall was an independent predictor of admission with pneumonia rather than with no pneumonia. Patients were almost twice as likely to have bacteremic pneumonia if the rainfall in the 14 days before admission was ≥125 mm.

Earlier literature, including that involving soldiers from the Vietnam War, suggests that inhalation is a common mode of infection with *B. pseudomallei* (13,14). This scenario was proposed for those exposed to dust raised by helicopter rotor blades in Vietnam (15). However, recent reviews have supported the predominant role of percutaneous inoculation of *B. pseudomallei* after exposure to muddy soils or surface water in endemic locations (10,11,16). Admissions with melioidosis pneumonia after presumptive inoculating skin injuries have been documented in patients with soil-contaminated burns (17) and are also common in our hospital (3,10). This finding suggests hematogenous spread to the lung rather than inhalation or spread from the upper respiratory tract. This finding is analogous to postprimary tuberculosis, with disease from hematogenous spread localizing in the upper lung zones, where highest alveolar oxygen tension exists (18). Moreover, septicemic melioidosis pneumonia patients are often more systemically ill than is suggested by initial chest x-ray, supporting the concept that bacteria spread to, rather than from, the lung.

Even if percutaneous inoculation is more common overall, the association of prior heavy rainfall with both pneumonia and more severe disease may well reflect a shift towards inhalation as the mode of acquiring *B. pseudomallei*. The periods of intense monsoonal rainfall are usually also associated with heavy winds and melioido-

Table 3. Multivariate analysis of predictors of clinical signs, symptoms, and outcome

| Clinical parameter | Independent predictors | Odds ratio (95% CI)[a] | p value |
|---|---|---|---|
| Pneumonia | Smoking | 2.51 (1.51 to 4.18) | <0.0001 |
| | Prior 14-d rainfall ≥125 mm | 1.58 (0.96 to 2.59) | 0.069 |
| | Kava use | 0.28 (0.09 to 0.82) | 0.020 |
| | Absence of risk factors | 0.29 (0.13 to 0.66) | 0.003 |
| Bacteremic pneumonia | Aboriginal ethnicity | 2.28 (1.33 to 3.90) | 0.003 |
| | Prior 14-d rainfall ≥125 mm | 1.81 (1.06 to 3.10) | 0.031 |
| | Absence of risk factors | 0.06 (0.01 to 0.48) | 0.007 |
| Septic shock | Prior 14-d rainfall ≥125 mm | 1.71 (0.99 to 2.97) | 0.057 |
| | Absence of risk factors | 0.07 (0.01 to 0.55) | 0.011 |
| Death | Prior 14-d rainfall ≥125 mm | 2.48 (1.32 to 4.66) | 0.005 |
| | Smoking | 1.93 (1.00 to 3.72) | 0.050 |
| | Absence of risk factors | No deaths in this group | <0.0001 |

[a]CI, confidence interval.

sis cases, and outbreaks are documented after cyclonic winds and rain (19,20). Aerosolization of bacteria from surface soil and water under such conditions is probable, resulting in the potential for inhalation of *B. pseudomallei*. Melioidosis following near-drowning is well recognized, with aspiration considered the likely infecting event, followed by pneumonia after an incubation period as short as 2 days (21–23).

That melioidosis can potentially be more severe after inhalation than after percutaneous inoculation is not surprising. This finding is well recognized for anthrax, plague, and tularemia and has implications for biological warfare considerations (24–26). However, as with melioidosis, septicemia with pulmonary involvement after percutaneous inoculation is well recognized with anthrax, plague, and tularemia. The lack of clarity of correlation between mode of infection, site of disease, and clinical course in the melioidosis literature is also evident in descriptions of the closely related disease, glanders (infection with *B. mallei*) (15).

Additional possible explanations for more severe disease after heavy rainfall include a larger bacterial inoculating dose and infection with more virulent bacteria. The association of melioidosis with the wet season has also been postulated to be due to movement of *B. pseudomallei* from deeper soil layers to the surface with the rising water table (27). Early studies also speculated that the increased isolation of *B. pseudomallei* from surface water after heavy rains resulted from increased growth of the bacteria (28). More recently, the possibility has been raised that *B. pseudomallei* may persist in the environment in a viable nonculturable state during times of stress, such as in prolonged dry seasons (20,29). Differential gene activation likely allows such environmental bacteria to respond and adapt to different environmental conditions (30). Recently, viable but nonculturable cells of *Francisella tularensis* have been shown to be avirulent in mice (31). Thus, both increased environmental bacterial load and increased virulence of environmental *B. pseudomallei* may possibly result from periods of heavy rainfall. A possible confounder to analyzing associations of rainfall with disease severity is that, whatever the mechanisms of more severe disease, such cases will tend to have shorter incubation periods. Therefore, the prior 14-day rainfall is likely to more closely reflect the rainfall associated with infection in these cases than in less severe cases, where incubation periods >14 days might occur.

In patients with melioidosis in this study, diabetes, alcohol excess, and chronic renal disease were associated with higher prior rainfall. We previously suggested that the predisposition to melioidosis in persons with these three conditions may relate primarily to impaired polymorphonuclear leukocyte (PMNL) functions (3). This hypothesis is supported by data from an observational, uncontrolled study showing improved survival with use of granulocyte colony-stimulating factor (G-CSF) in melioidosis septic shock (32). Recent animal data suggest an important role for lung-derived G-CSF in controlling intrapulmonary infection (33). Therefore, in diabetes, alcoholism, or chronic renal disease, both impaired phagocytic activity of alveolar macrophages and impaired recruitment of PMNL into the lungs as a result of acquired dysfunction of alveolar macrophages may be critical, in addition to impaired PMNL function, in determining the predisposition to melioidosis pneumonia. Such a predisposition is likely to be especially important in influencing whether infection becomes established after inhalation of *B. pseudomallei*. The association of diabetes, alcohol excess, and chronic renal disease with higher prior rainfall may therefore reflect a particular susceptibility to inhalation as a mode of infection in patients with these risk factors. Alternatively, this finding may reflect a greater influence of bacterial load or organism virulence in these risk groups. Either explanation is consistent with the observation from Thailand that risk factors and level of environmental exposure to *B. pseudomallei* have a compound interaction, as is evident in the especially high rates of melioidosis in diabetic rice farmers (34).

We have shown that the intensity of rainfall in the 14 days before a person is admitted to a hospital with melioidosis is an independent predictor of the patient's having pneumonia, septic shock developing, and death. We postulate that this may reflect a shift towards inhalation of *B. pseudomallei* as the mode of transmission after heavy monsoonal rains and winds.

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Dr. Currie is an infectious diseases physician at Royal Darwin Hospital, head of the Infectious Diseases Program at the Menzies School of Health Research in Darwin, and head of the Biomedical Program of the Australian government–funded Cooperative Research Centre for Aboriginal and Tropical Health. His clinical and research interests focus on tropical diseases in northern Australia and the region, including melioidosis, rheumatic fever and streptococcal infections, scabies, and snakebite and jellyfish toxinology.

Ms. Jacups is a clinical and public health nurse with experience working in remote indigenous Australian communities. She coordinates data collection and analysis for the prospective Darwin melioidosis study.

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Address for correspondence: Bart Currie, Menzies School of Health Research, P.O. Box 41096 Casuarina, Northern Territory 0811, Australia; fax: 61-8-89275187; email: bart@menzies.edu.au

# Comparative Molecular and Microbiologic Diagnosis of Bacterial Endocarditis

**Isabelle Podglajen,*† Fabienne Bellery,* Claire Poyart,‡ Philippe Coudol,* Annie Buu-Hoï,* Patrick Bruneval,* and Jean-Luc Mainardi*†**

Sequencing of 16S rDNA, and of *sodA*$_{int}$ and *rpoB*$_{int}$ in some cases, was applied to DNA from heart valves of 46 patients (36 with definite and 10 with possible endocarditis). Sequence-based identifications were compared with those obtained with conventional methods. Among the 36 definite cases, 30 had positive blood cultures and 6 had negative cultures. Among the 30 positive cases, sequencing of 16S rDNA permitted identification of species (18), genus (8), or neither (4); *sodA*$_{int}$ and *rpoB*$_{int}$ sequencing was necessary for species identification in 8 cases. Species identifications were identical in only 61.5%, when conventional techniques and DNA sequencing were used. In five of the six blood culture–negative endocarditis cases, sequencing identified *Bartonella quintana* (3), *B. henselae* (1), and *Streptococcus gallolyticus* (1). Our results demonstrate a clear benefit of molecular identification, particularly in cases of blood culture–negative endocarditis and of possible endocarditis, to confirm or invalidate the diagnosis. Moreover, in 19.4% of the definite cases, the improvement in species identification by sequencing led to improved patient management.

According to the earliest published report on the subject, the prevalence of blood culture-negative endocarditis once ranged from 2.5% to 31% (1). In more recent studies, approximately 9% is the reported rate (2). One explanation for the improvement in the bacteriologic diagnosis of endocarditis is better knowledge of its clinical symptoms and risk factors, which has encouraged earlier blood culture. Another reason is the improvement in bacterial culture techniques, with prolonged incubation times, presence of carbon dioxide, enriched culture media, and timed subcultures. Thus, the isolation of fastidious microorganisms including *Abiotrophia* (new genus *Granulicatella*) and the HACEK group, has improved dramatically, and organisms frequently missed with the use of earlier blood culture techniques are now recognized. Another improvement is the use of specific serologic tests for certain microorganisms. Such tests, associated with cell cultures, are now recommended for patients with blood culture-negative endocarditis for which *Coxiella burnetii* and *Bartonella* spp. are the suspected causative organisms (3–5). Despite these improvements, the diagnosis of blood culture–negative endocarditis remains a challenge. The absence of positive culture is most frequently due to previous antimicrobial drug treatment or to bacterial species that are difficult to grow or that remain nonculturable in the laboratory.

To overcome these problems, molecular techniques using broad-range DNA primers for amplification of bacterial 16S rDNA directly from clinical samples and subsequent nucleotide sequencing (6,7) have been proposed to establish the infectious etiology (8–10). This approach, combined with sodA$_{int}$, encoding superoxide dismutase (11) and rpoB$_{int}$, encoding the β sub-unit of RNA polymerase (12) sequencing when 16S rDNA sequences were not sufficiently discriminating, was used here 1) to evaluate the bacterial content of 46 resected heart valves from patients operated on for endocarditis, 2) to compare the results with bacteriologic and histologic findings from heart valves and from preceding blood cultures, and 3) to analyze the data with respect to the clinical background of the patients, including the modified Duke criteria (13).

## Patients and Methods

### Patients

From October 2000 to June 2002, all patients operated on for endocarditis at the Hôpital Européen Georges Pompidou, Paris, were classified according to the modified Duke criteria for the diagnosis of infective endocarditis (13). We studied a total of 46 cases (26 men and 20 women;

*Hôpital Européen Georges Pompidou, Paris, France; †INSERM E0004, Université Paris VI, Paris, France; and ‡Hôpital Necker, Université Paris V, Paris, France

average age 55.5 years; range 20–86 years), including 36 clinically definite (31 native valves and 5 prostheses) and 10 clinically possible (5 native valves and 5 prostheses) cases. Among the 36 definite cases, 27 patients had two major criteria, and 9 patients had one major and three minor criteria. All 10 patients classified as having possible endocarditis had one major and one minor criterion. Thirty-two (69.5%) of the 46 patients had been transferred to our hospital for surgery. In these cases, blood cultures and conventional bacterial identification had been performed in other hospitals (23 hospitals, including 3 in foreign countries). Twenty-five patients without endocarditis who were operated on for valve replacement were studied as controls.

### Microbiologic Methods

The excised heart valves were processed under a laminar flow hood. Portions of abnormal valve tissue were ground with a mortar and pestle and cultured on Columbia sheep blood agar, and chocolate agar supplemented with IsoVitaleX (bioMérieux, Marcy l'Etoile, France) (at 37°C aerobically and with 5% CO2 for 10 days), Schaedler sheep blood agar (at 37°C anaerobically for 10 days), brain heart infusion broth, and brain heart infusion broth supplemented with IsoVitaleX (aerobically at 37°C for 30 days). In each case, a valve culture was also performed in an anaerobic blood culture vial (Vital, bioMérieux), which was incubated for 1 month at 37°C. A direct Gram (and Giménez if necessary) stain was performed. Bacteria from isolated colonies were identified according to standard procedures (14). Heart valve samples were stored at –80°C before DNA extraction. When bacteria were isolated from blood cultures in our hospital, they were identified according to standard procedures (14).

### Molecular Methods

#### DNA Extraction

DNA extractions and polymerase chain reactions (PCR) were carried out in separate areas. DNA from heart valve material was extracted (two parallel extractions per valve), according to the manufacturer's instructions, with the QIAMP Tissue Kit (Qiagen, Courtaboeuf, France). For each batch of extraction, a negative extraction control containing all reagents except heart valve material was included.

#### PCR Amplification

For each specimen, DNA in 2 V of crude extract (5 µL and 20 µL) was directly amplified with one or two sets of primers (p13B and p91E or p13B and p515FPL), as described previously for 16S rDNA (15), with *d1* and *d2* for $sodA_{int}$ (11) and with $CM_7$ and $CM_{31b}$ for $rpoB_{int}$ (12). Reaction mixes (50 µL) were set up with two Taq DNA polymerases (Superpak, Sigma, St. Louis, MO, and Qbiogen, Illkirch, France), according to the specifications of the manufacturers, and 0.2 µM of each appropriate primer. For each batch of six samples, two negative controls were included. PCR was performed in a PTC 200 thermocycler (VWR International, Fontenay-sous-Bois, France) with the following thermal cycling parameters for 16S rDNA: 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. When the PCR result was negative, an amplification of the human β-globin gene was performed as an internal extraction control. Sequence analysis of both strands was carried out on a 3700 DNA analyzer (Applied Biosystems, Courtaboeuf, France). Similarity searches were carried out against GenBank and the Ribosomal Database Project (RDP II, Michigan State University, East Lansing, MI).

#### Histologic Analysis

Pieces of formalin-fixed abnormal valve tissue were examined. Paraffin sections were cut and stained with hematoxylin and eosin and Gram (and Giménez if necessary) stains. The histopathologic diagnosis was based on valvular inflammation, vegetation, microorganisms, or other features consistent with endocarditis (16).

## Results

Among the 46 cases of endocarditis, 36 were identified before surgery as definite and 10 as possible, according to the modified Duke criteria (13).

### Definite Endocarditis

Among the 36 cases of definite endocarditis, blood cultures were positive in 30 and negative in six (Table). After surgery, histopathologic criteria were present in all clinically definite cases.

#### Blood Culture–Positive Patients

PCR amplification of the bacterial 16S rDNA directly from the resected valves of the 30 patients with positive blood cultures was positive in 26 cases and negative in 4 (Table). The mean duration of antimicrobial drug treatment before surgery was 24.6 days (range 1–75 days) for the PCR-positive group and 34.5 days (range 18–53 days) for the PCR-negative group. In the negative group, three patients had right-side endocarditis stemming from pacemaker infection attributable to *Staphylococcus epidermidis*, *S. caprae,* or an association of *S. aureus*, *Escherichia coli,* and *Streptococcus pneumoniae*, respectively. The fourth patient had endocarditis on the mitral valve attributable to *Staphylococcus aureus*. The direct examination of valve samples after Gram staining showed evidence of bacteria in two cases (*S. aureus* and *S. caprae*); culture was positive in only one, yielding *S. caprae*.

Table. Comparison of results obtained from blood cultures with conventional methods of identification (CMI-BC) and from valves with sequence-based identification (SBI-V)

| Positive blood culture (n = 30) | | | | Negative blood culture (n = 6) | |
|---|---|---|---|---|---|
| PCR positive (n = 26) | | | PCR negative (n = 4)[a] | PCR positive (n = 5) | PCR negative n = 1 |
| Species identification | | | | | |
| Concordance (n = 16) | Discordance (n = 10) | | | | |
| (CMI-BC/SBI-V) | CMI-BC | SBI-V | CMI-BC | SBI-V | |
| *Strep. mutans* | *Strep. agalactiae* | *Lactobacillus crispatus* | *Staph. aureus* | *Strep. gallolyticus* | |
| *Strep. sanguis* | *Gemella spp.* | *Aerococcus urinae* | *Staph. epidermidis* | *Bartonella quintana* (3) | |
| *Strep. gallolyticus* (4) | Group C streptococci | *Abiotrophia adiacens* | *Staph. caprae* | *B. henselae* | |
| *Campylobacter fetus* | *Strep. mitis* (2) | *Strep. sanguis* (2) | | | |
| *Escherichia coli*[b] | *Strep. sanguis* | *Strep. oralis*[c] | *Staph. aureus* + | [d] | |
| *Propionibacterium acnes* | *Strep. salivarius* | *Strep. oralis*[c] | *Strep. pneumoniae* | | |
| *Staph. aureus* (2) | *Haemophilus influenzae* | *H. aphrophilus* | + *E. coli* | | |
| *Strep. oralis* (2)[c] | *H. aphrophilus* | *H. paraphrophilus* | | | |
| *Strep. mitis* (2)[c] | *Streptococcus* spp. | *Strep. gordonii* | | | |
| *Strep. pneumoniae*[c] | | | | | |

[a]In the case of positive blood cultures with *Staphylococcus (Staph) aureus*, *Escherichia coli,* and *Streptococcus (Strep) pneumoniae*, PCR was positive, but direct sequencing was not interpretable.
[b]Species identification based on sequence analysis of PCR-amplified $rpoB_{int}$.
[c]Species identification based on sequence analysis of PCR-amplified $sodA_{int}$.
[d]Three species were identified in the blood culture.

From the 26 valves yielding positive PCR, 5 were culture positive, with the same microorganism as previously identified in the blood cultures (*Escherichia coli*, *S. aureus* [2], *Propionibacterium acnes,* and *Streptococcus mitis*). The mean duration of antimicrobial drug treatment was 8.2 days (range 1–22 days) when the valve culture was positive and 30.2 days (range 5–75 days) when the valve culture was negative. There was full agreement in 16 cases (61.5%) between the bacterial identifications obtained with conventional methods after blood culture and sequence-based identifications with DNA extracted from heart valves (Table). Discrepancies (38.5%, Table) were due to misidentification at the genus level in three cases, misidentification of the species in six cases, and absence of identification to the species level after blood culture in one case. Overall, there were seven cases in which only $sodA_{int}$ sequence analysis allowed differentiation between *S. mitis*, *S. oralis* and *S. pneumoniae* and one case in which *rpoB* analysis allowed differentiation between *E. coli* and other *Enterobacteriacae*.

### Blood Culture–Negative Patients

In the six cases of negative blood culture (Table), the patients had been treated with antimicrobial drugs before surgery; culture of their valves was also negative. The mean duration of antimicrobial drug treatment was 60 days (range 45–120 days). In five of the six patients, 16S rDNA sequencing permitted bacterial identification from the resected valves. Four of these contained *Bartonella* (three *B. quintana* and one *B. henselae*). Three of the patients with *Bartonella* endocarditis were transferred directly from Africa to our hospital for surgery; primary diagnosis was performed by PCR and confirmed by subsequent serology. In the fourth patient, serologic results were positive before surgery and confirmed by PCR. Serologic testing with a microfluorescence assay showed titers of $\geq$1:800 for immunoglobulin G antibodies (5). *S. gallolyticus* was identified in the fifth patient, who had been treated with amoxicillin for a suspected urinary tract infection before the blood cultures were taken. In the sixth patient (PCR negative), the histologic results showed a subacute aortic endocarditis with a single epithelioid granuloma, the cause of which remained undetermined.

### Possible Endocarditis

Ten patients were classified before surgery as having possible endocarditis (13). In these patients, blood cultures were negative, and PCR did not indicate a microorganism. The histologic analysis of the resected valves also did not indicate any feature of endocarditis, in accordance with the bacteriologic results.

### Control Patients

Twenty-five patients who were operated on for valve replacement but who did not have endocarditis were included as controls. PCR from resected valves and histopathologic signs were negative in all these patients.

## Discussion

The clinical diagnosis of infective endocarditis, particularly in patients who have negative blood culture, were previously treated with antimicrobial drugs, or both, is generally difficult. In this study of 46 cases of definite or possible endocarditis, we used amplification of 16S rDNA extracted from valves and subsequent sequencing to identify the bacterial agent responsible for endocarditis. The

results were compared with those obtained with conventional bacteriologic methods of identification after blood culture. When species identifications based on 16S rDNA sequencing were ambiguous, PCR amplification and sequencing of $sodA_{int}$ or $rpoB_{int}$ (in one case) were also performed.

When we considered the cases of definite endocarditis with positive blood cultures (Table), there was an agreement of 30% (if one includes the PCR-negative results) between the bacterial identifications obtained after sequencing of 16S rDNA extracted from heart valves and those obtained with conventional techniques after blood culture. There was agreement of 53.3% when the molecular identification included the analysis of $sodA_{int}$ to differentiate between *S. mitis*, *S. oralis,* and *S. pneumoniae* (11) and of $rpoB_{int}$ to differentiate between *E. coli* and closely related *Enterobacteriacae* (12). Combined sequence analysis entailed the refinement of genus to species identification in one case, the revision of species identification in six, and the revision of genus identification in another three. In four cases (13.3%), the PCR result was negative despite positive blood culture (with positive valve culture in one of them) and histologic results that showed features suggestive of endocarditis. This finding was most likely due to the workup of inadequate fractions of the valves (i.e., in some PCR-positive cases, a positive reaction was obtained only with one of the two valve fractions). Multiple fractions should therefore be selected after meticulous macroscopic examination. PCR inhibitors were not likely present since the control reaction with the β-globin gene was positive in all cases. Obviously, no identification can be expected from direct sequencing of PCR products in the presence of multiple bacterial species, as in one case encountered in this study.

If species, as opposed to genus identification, may have only modest consequences on the management of most patients, for some cases the consequences can be substantial. Here, the identification of *Lactobacillus crispatus,* instead of *S. agalactiae*, led to the search and treatment of the dental portal of entry and the identification of *Abiotrophia adiacens,* instead of group C streptococci; thus in this last case, the antimicrobial drug treatment was prolonged. In five of six cases of negative blood culture, PCR permitted the identification of the responsible microorganism (Table). The identification of *S. gallolyticus* in one patient led to the search for and removal of a precancerous intestinal polyp. In four cases, *Bartonella* species were identified and the antimicrobial drug regimens modified with the introduction of gentamicin (17) and prolonged treatment. 16S rDNA PCR is of particular diagnostic value when serology has not been performed and when a serologic test is not available (as in the case of *Tropheryma whippleii*). Moreover, we believe that PCR on resected valves should be performed in patients with positive blood cultures, under two conditions: 1) in the absence of species identifications; or 2) if there is a lack of correlation between the putative microbial identification by conventional microbiologic techniques and the clinical signs and symptoms or course of the endocarditis.

Our study shows that analysis of 16S rDNA extracted from valves is not beneficial only in cases classified as definite endocarditis. It can also serve as a valuable diagnostic tool to confirm or rule out the suspicion of possible endocarditis. The results obtained with this method in the 10 cases we describe were in full agreement with the histologic findings, which did not indicate features of infective endocarditis. All patients for whom negative results were obtained with conventional and molecular methods were secondarily reclassified and were rejected following the Duke's scheme (13). As a therapeutic consequence, the use of unjustified antimicrobial drug treatment was stopped.

We conclude that the diagnosis of bacterial endocarditis may benefit from adding molecular biologic identification to conventional identification after standard cultures. This finding is in agreement with those from a recent study that analyzed a group of patients similar in size and with endocarditis due to a comparable set of infectious agents (18) as well as with our recent observation of *C. burnetii*, *Staphylococcus lugdunensis,* and *T. whipplei* in culture-negative valves from endocarditis patients with negative blood cultures. While no false-positive results were obtained with the PCR-based approach in our study, few were false negative. On the other hand, this approach contributes to an improvement in bacterial identification in a substantial number of cases as well as improvement in patient management in approximately 20%.

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Dr. Podglajen is a microbiologist working in the Microbiology Department at the Hôpital Européen Georges Pompidou, Paris, France. Her research interests include detection of noncultivable pathogens and mechanisms of dissemination and expression of resistance genes in bacteria.

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Address for correspondence: Jean-Luc Mainardi, Unité Mobile de Microbiologie Clinique, Service de Microbiologie Clinique, Hôpital Européen Georges Pompidou, 20 Rue Leblanc, 75015 Paris, France; fax: 33-1-56-09-24-46; email: jlmainar@bhdc.jussieu.fr or jean-luc. mainardi @hop.egp.ap-hop-paris.fr

# Emerging Genotype (GGIIb) of Norovirus in Drinking Water, Sweden

**Karin Nygård,* Maria Torvén,* Camilla Ancker,† Siv Britt Knauth,† Kjell-Olof Hedlund,* Johan Giesecke,* Yvonne Andersson,* and Lennart Svensson*‡**

From May through June 2001, an outbreak of acute gastroenteritis that affected at least 200 persons occurred in a combined activity camp and conference center in Stockholm County. The source of illness was contaminated drinking water obtained from private wells. The outbreak appears to have started with sewage pipeline problems near the kitchen, which caused overflow of the sewage system and contaminated the environment. While no pathogenic bacteria were found in water or stools specimens, norovirus was detected in 8 of 11 stool specimens and 2 of 3 water samples by polymerase chain reaction. Nucleotide sequencing of amplicons from two patients and two water samples identified an emerging genotype designated GGIIb, which was circulating throughout several European countries during 2000 and 2001. This investigation documents the first waterborne outbreak of viral gastroenteritis in Sweden, where nucleotide sequencing showed a direct link between contaminated water and illness.

Viruses have emerged as important causes of foodborne and waterborne diseases in recent years, with numerous outbreaks associated with Norwalk viruses. This virus is the prototype in the genus *Norovirus,* family *Caliciviridae*, which includes a large number of genetically related strains associated with acute gastroenteritis. Longitudinal surveys have shown that caliciviruses and especially noroviruses are common causes of nosocomial and community-associated outbreaks of acute gastroenteritis worldwide (1–5). Norovirus-associated gastroenteritis is transmitted by the fecal-oral route. It occurs both as sporadic community cases and as large outbreaks in, for example, nursing homes, hospitals, schools, and ships. The outbreaks often are associated with ingestion of food or contaminated water. Norovirus-associated waterborne outbreaks (6) have been associated with contamination of septic tanks, industrial water system (7–9), and swimming water (10–12) as well as drinking contaminated drinking water (13–18).

We describe a waterborne outbreak caused by contaminated drinking water. While no pathogenic bacteria were found in collected samples, identical noroviruses belonging to genogroup II (GGIIb) were identified in both stool and water samples.

## Methods

### Outbreak Description

An outbreak of acute gastroenteritis occurred in a combined activity camp and conference center in Stockholm County from May to the end of June 2001. During the summer, the center caters to both overnight guests and daytime visiting groups. A separate cafe for outside visitors to the nearby beach is also on the premises. Environmental and microbiologic investigations were conducted to determine the source of the outbreak and implement control measures to stop the outbreak and prevent similar situations in the future.

### Environmental Investigation

The municipal environmental health unit was first contacted on June 12. The facilities were inspected, and water and food samples were collected. On June 15, the Stockholm County Council Department of Communicable Disease Control and Prevention was contacted, and the premises were reinspected on June 25 and July 3. Additional water samples were taken on several occasions during June and July.

### Microbiologic Investigation

#### Bacteriologic Investigation

A total of 11 stool specimens were collected (2 from staff and 9 from visiting guests) and cultured for bacterial enteropathogens, including *Salmonella, Shigella, Campylobacter,* and *Yersinia*. Ten water samples were

*Swedish Institute for Infectious Disease Control, Solna, Sweden; †Karolinska Hospital, Stockholm, Sweden; and ‡University of Linköping, Linköping, Sweden

examined for fecal coliforms, total coliforms, fecal streptococci, and sulphite-reducing clostridia. Seven food products were examined for aerobe microorganisms, enterobacteriaceae, enterococci, fecal coliforms, *Salmonella, Bacillus cereus*, C*lostridium perfringens,* coagulase-positive staphylococci, yeast, and mold. Approved standard laboratory methods were used for all bacteriologic investigations.

### Virologic Investigation

Stool samples were examined for norovirus by electron microscopy and reverse transcription–polymerase chain reaction (RT-PCR), as previously described (4,19,20). Briefly, viral RNA was extracted from 100 µL of a 10% stool suspension with the guanidine thiocyanate–silica extraction method (21) followed by RT-PCR with primer pair JV12/JV13, which yields a 326-bp product, located in the gene for RNA-dependent RNA polymerase.

Three water samples collected from the kitchen, the water works, and the public beach were tested for norovirus. These water samples were concentrated by a method slightly modified from Gilgen et al. (22). Briefly, 0.5 L of water was filtered through a positively charged 0.45-µm membrane (Zetapor, Millipore Corp., Bedford, MA) followed by virus elution from the membrane with 50 mM glycine–NaOH, pH 9.5, containing 1% beef extract as described (16). A Centricon-100 microconcentrator (Amicon, Millipore) was used for further concentration to 100 µL.

For the water samples, a nested PCR was used. RNA-extraction and first-round PCR were performed as described in this section. For the nested PCR, new inner primers were designed from alignment of sequences circulating in Sweden and sequences from the GenBank database. The inner primers were designated n12 (5'-TGG GAY TCM ACD CA-3') and n13 (5'-CTT CAG ANA GNG CAC ANA GAG T-3'). These primers yield a 234-bp product.

### Nucleotide Sequencing

The PCR products from two human and two water samples were sequenced. The samples were sequenced from both directions by using primer pair n12/n13 (water samples) and primer pair JV12/JV13 (patient samples) by ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) on an ABI 310 automated sequencer. Sequences from prototype strains of caliciviruses from the GenBank database were aligned with the sequences from patient and water samples. Programs from the PHYLIP program package (National Institutes of Health, Besthesda, MD) were used to construct the phylogenetic trees. SEQBOOT (NIH) was used for bootstrap resampling to produce 100 different datasets from the aligned sequences. From these datasets, phylogenies were estimated by DNAMLK (NIH). CONSENCE (NIH) was used to construct a consensus tree from the obtained data and to obtain bootstrap values. The tree was drawn with Treeview (Page RD. TREEVIEW, University of Glasgow, Glasgow, Scotland). The nucleotide sequence accession number assigned by GenBank is AY240939.

## Results

### Environmental Investigation

The activity camp, conference center, and nearby cafe were supplied with ground water from their own private wells, located at the premises. Six months before the outbreak, they had started to use water from two newly drilled wells located within 20 m of each other. Only chemical parameters had been analyzed before the new wells were put in use. The water from both wells was held in a common reservoir and was not disinfected before distribution. According to personnel at the camp, the wells were approximately 80 m deep, and the soil layer was 18 m at the location of the wells. A third well was drilled at the same time and located close to the other two but was not put in use. Previously, water had been obtained from an old well located further away from the facilities. Since this old well had limited capacity, and sometimes its water was not potable, new wells with enough capacity to fulfill increased demands had been drilled. For practical and economical reasons, the new wells had been placed closer to the center facilities.

Sewage from the camp was connected to the community system and was transported to the nearest sewage treatment facility. The sewage pipes were old, and personnel reported that on several occasions problems with the capacity of the system had occurred. In April 2001, a blockage of the overflow in the low-pressure-system well, located near the kitchen facilities, occurred, and sewage had spilled out on the ground. On this site, located approximately 100 m from the ground water wells, the rock was covered by only 1–2 m of soil. Sewage had also overflowed on the ground near the kitchen in the autumn 2000 because of a stoppage in the sewage pipeline connection to the community system.

### Epidemiologic Investigation

Approximately 200 people contracted gastroenteritis after consuming tap water. They had clinical symptoms of vomiting, diarrhea, abdominal pain, and fever (mostly a combination of these symptoms). Duration of symptoms varied from several hours to 2 to 3 days. The first known cases of illness occurred in a group of adults participating in a 1-day conference on May 31. Of 16 persons (all adults), 8 became ill (attack rate 50%) with gastrointestinal

symptoms. Nearly 2 weeks later (June 9–10), a school class with 28 pupils (8–13 years of age) arrived for an overnight stay; approximately half became ill (attack rate 50%) with similar symptoms. The following day (June 10), the first participants of a sport-training camp arrived. The camp lasted for 10 days, during which a total of 150 children (9–12 years of age) and 20 adults stayed at the facilities in three overlapping periods. The first cases of illness in this group occurred the day after arrival; approximately 100 persons became ill (attack rate 58%). During the next 2 weeks, several more guests and visiting groups reported illness after visiting the center; some of these persons had not eaten but had just drunk the center's tap water. Two of these groups were children (8–13 years of age); the attack rate in both groups was 40%. The outbreak was not controlled until the facilities closed for >1 week in the end of June. Some of the personnel working at the center also reported gastrointestinal symptoms, including one of the kitchen personnel, who became ill on June 13 and was taken off duty.

### Control Measures

On the first visit, general recommendations regarding kitchen hygiene and cleaning of the environment were given. When the results of the first water samples were ready, additional recommendations on boiling all water used for drinking and food preparation were given. At the same time, the environment was thoroughy sanitized. In spite of these measures, new cases continued to occur, so the facilities were closed for >l week at the end of June to interrupt possible continuous transmission among guests. After this measure, no new cases occurred. Different alternatives to prevent similar situations in the future were discussed, and the decision was made to close the wells and connect to the municipal water supply.

## Microbiologic Investigation

None of the stool samples collected from the two staff or nine visitors were positive for *Salmonella, Shigella, Campylobacter,* or *Yersinia,* nor were any viruses other than calicivirus found by electron microscopy. Of the 11 samples examined by norovirus-specific PCR, 8 had an amplified PCR product of the expected size. No foodborne pathogens were found in any of the food items investigated. The first samples were collected from tap water in the kitchen on June 12 and water collected from the water works on June 18 showed strong indication of fecal contamination (Table). Samples collected from the wells 1 and 3 on June 20 and 27 showed evidence of fecal contamination, as did sampling of well 2 in July (Table). Water samples from the tap in the kitchen and the water works, collected on June 18, were positive for norovirus with a nested PCR and showed evidence of fecal contamination (Table). The water samples collected from the beach were negative for norovirus. PCR amplicons from two visitors (samples collected at different time points) and the two positive water samples were sequenced and compared. The strains were identical to each other and identical to strain "Gothenburg" (Figure) and had 97%-98% nucleotide identity to Spanish GGIIb strains (AJ487474, AJ487794, AJ487795, AJ487789, AJ487794) (23).

## Discussion

We describe an epidemiologic and microbiologic investigation of a waterborne outbreak in which at least 200 persons became ill after staying at a combined activity camp and conference center in the Stockholm area. A large number of daytime visitors to the beach and nearby cafe may also have become ill, so the actual number of cases has likely been underestimated. The visitors in different groups did not eat the same food items, and some visitors did not eat any food. Several of the short-stay visitors consumed only camp tap water, which was fecally contaminated. The source of illness was drinking water obtained from ground water wells that had been contaminated by sewage. Person-to-person transmission and transmission through contaminated surfaces probably contributed to the rapid spread among the overnight visitors. While no pathogenic

Table. Results from bacteriologic analysis of water samples, Sweden, 2001

| Place | Date (2001) | Heterotrophs/ mL (2 d) | Coliforms/ 100 mL | *E. coli*[a]/ 100 mL | Sulphite-reducing clostridia/100 mL | Fecal streptococci /100 mL |
|---|---|---|---|---|---|---|
| Tap water, kitchen | 6/12 | 80 | 140 | 47 | - | - |
| Water works | 6/18 | 690 | 100 | 32 | <1 | 2 |
| Tap water, kitchen | 6/18 | 530 | 130 | 40 | <1 | 1 |
| Well 1 | 6/20 | >300 | 430 | >100 | - | - |
| Well 2 | 6/20 | >300 | 1 | <1 | - | <1 |
| Well 3 | 6/27 | 2,100 | 19 | 1 | - | - |
| Old well | 6/27 | 1,100 | 630 | 22 | - | - |
| Storm water | 6/27 | 2,000 | 190 | 3 | - | - |
| Well 2 | 7/03 | 1,300 | 160 | 6 | - | - |
| Beach | 7/17 | 16,000 | 1 | - | - | - |

[a]*Escherichia coli.*

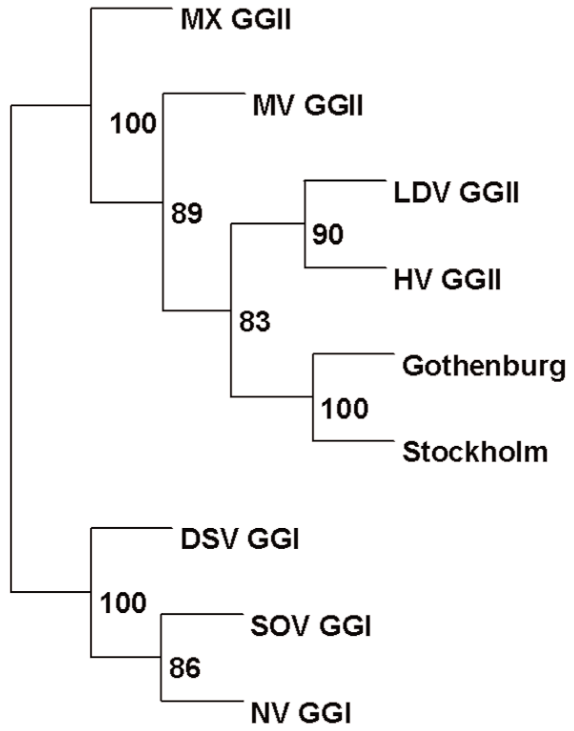


Figure. Phylogenetic tree based on a 198-nt region of the gene coding for RNA-dependent RNA-polymerase (located in ORF1), showing patient and water samples and some prototype strains of calicivirus from the GenBank database (accession no.: MX, Mexico U22498; MV, Melksham X81879; HV, Hawaii U07611; LDV, Lordsdale X86557; DSV, Desert Shield U04538; SOV, Southampton L07418; NV, Norwalk M87661; Gothenburg AF365989). Bootstrap values are given in percentage at the nodes.

bacteria were found in water or stool samples, norovirus belonging to genogroup II with identical nucleotide sequence in the polymerase region was obtained from both stool and water samples. The strain was identical to strain Gothenburg, previously identified in Sweden and belonging to the emerging genotype cluster GGIIb. These strains have circulated in several European countries during 2000 and 2001 (23). While the GGIIb outbreak in this study was associated with contaminated water, previously reported GGIIb strains have been associated with school, nursing home, and rural village outbreaks (23). That all were identified during 2000 and 2001 further supports the hypothesis of an emerging strain or cluster of strains.

The drinking water was obtained from deep ground wells close to the cafe. Before the outbreak, this cafe had had problems with low pressure in its well, which caused blockage of the sewage system. As a consequence sewage spilled out and lead to contamination of the environment. At the contamination site, the soil was only 1–2 m deep, and cracks in the rock may have facilitated migration of microorganisms from the sewage to the ground water. Norovirus can migrate through soil and contaminate well water and cause gastroenteritis outbreaks (7,24).

One possible explanation for the protracted duration of the outbreak could be a continuous leak from the sewage system, which would have caused persistent contamination of the environment. The ill persons staying at the facilities might have contributed to increased viral load in the sewage, and problems with the sewage collection system would then have further aggravated contamination of the water supply. Another possibility was that the water initially caused the outbreak, but person-to-person spread contributed to the continuous transmission.

The low infectious dose of norovirus readily allows transmission through environmental contamination and aerosols. Boiling the water used for drinking and food preparation was recommended. Since the risk for transmission through aerosols generated when showering with possibly contaminated water is not well established, no recommendations were made in this regard. Another problem was how to decontaminate bed linen and other fabrics. Washing at high temperatures is the recommended procedure to eliminate viral contamination. However, if the water used for washing is contaminated, the rinsing process may lead to recontamination of the fabrics. We recommended boiling or heating water for washing to >90°C in the presence of detergents.

This outbreak illustrates some problems related to private water supply. In Sweden, approximately 15% of the population has a private water supply, and the extent of gastrointestinal illness related to water is not clearly identified. Problems with person-to-person transmission of noroviruses are well known; however, risks related to exposure through contact with contaminated water and environment through vomit and aerosols are not well established.

In summary, detecting identical virus in both drinking water and stool specimens from ill persons strongly indicated that norovirus was the principal pathogen of this outbreak. Nucleotide sequence analysis identified a norovirus designated GGIIb (23).

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Dr. Nygård is an epidemiologist. Her areas of interest are epidemiology, water control, and foodborne and vector-borne infectious diseases.

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Address for correspondence: Lennart Svensson, Department of Molecular Virology, University of Linköping, 581 85 Linköping; fax: +46 13 22 47 89; email: lensv@imk.liu.se

Many believe that art and science, if coordinated in some way, might expose wider vistas of natural truth. After all, the aim of artist and scientist alike is to communicate a new and valuable way of regarding the natural world around us.

**–Graeme L. Stephens**
In "The Useful Pursuit of Shadows"

# *Mycobacterium tuberculosis* Beijing Genotype

**Troels Lillebaek,* Åse B. Andersen,† Asger Dirksen,‡ Judith R. Glynn,§ and Kristin Kremer¶**

Molecular epidemiologic studies of strains of *Mycobacterium tuberculosis* are currently conducted worldwide. The genetically distinct Beijing family of strains has been associated with large outbreaks of tuberculosis, increased virulence, and multidrug resistance. However, in this first population-based search for Beijing strains in the Danish DNA fingerprint database, analysis of 97% of all culture-positive tuberculosis patients in 1992 to 2001 showed that 2.5% of 3,844 patients, 1.0% of Danish-born patients, and 3.6% of immigrants (from 85 countries) had Beijing strains. No Beijing strains were found among 201 strains from Danish-born patients sampled in the 1960s, and no evidence of an increase in Beijing strains was found over time. The true prevalence of Beijing strains worldwide is unknown because only a fraction of global strains have been analyzed.

New technologies have enabled researchers to clarify fundamental questions about the epidemiology and pathogenesis of tuberculosis that were previously obscure (1). Although the *Mycobacterium tuberculosis* genome is genetically highly conserved, insertion sequences, repetitive elements, genomic deletions, and single nucleotide polymorphisms cause genetic polymorphisms. These polymorphisms can be visualized by various genotyping techniques, often referred to as DNA fingerprinting, whereby specific strains of *M. tuberculosis* can be characterized on the basis of their DNA patterns (2). Restriction fragment length polymorphism (RFLP) typing by using the insertion sequence IS*6110* as a probe for strain differentiation is the most widely applied DNA fingerprinting method to study the epidemiology of tuberculosis (1). This technique has been used for population-based transmission surveillance (1), including studies across national boundaries (3). In connection with this effort, one genetically highly conserved group of strains of *M. tuberculosis* collectively known as "the Beijing family" has attracted special attention (2,4). These strains are reported to be highly prevalent throughout Asia and in the countries of the former Soviet Union (5–9); they may possess selective advantages compared with strains of other *M. tuberculosis* genotypes (5); and they are sometimes associated with multidrug resistance (6,8,10,11) and with specific pathogenic properties and increased virulence (6,8,12). Furthermore, Beijing family strains may be increasing in frequency and be spreading to new geographic areas (5,10,11,13). The "W-strain family" concurrently identified on the North American (10) and Asian continents (5) is part of the Beijing family. In this study we investigated the Beijing strain family in Denmark.

*National Institute for Prevention and Control of Infectious Diseases and Congenital Disorders, Copenhagen, Denmark; †Rigshospitalet University Hospital, Copenhagen, Denmark; ‡Gentofte University Hospital, Gentofte, Denmark; §London School of Hygiene and Tropical Medicine, London, United Kingdom; and ¶National Institute of Public Health and the Environment, Bilthoven, the Netherlands

## Methods

### Data Collection

Microbiologic analyses of mycobacteria have been carried out at the International Reference Laboratory of Mycobacteriology at Statens Serum Institut in Copenhagen since 1922. This is the only laboratory that performs culture-based tuberculosis diagnosis for the Danish Kingdom. Since 1992, DNA fingerprinting of strains of the *M. tuberculosis* complex has been implemented on a nationwide scale by using the internationally standardized method of IS*6110* RFLP typing (14). Fingerprints from a total of 4,102 strains from 3,844 patients were available for the current study, representing 97% of culture-positive patients in Denmark in 1992 to 2001. When more than one strain was available, the earliest specimen was included in the analysis. In addition, a search for Beijing family strains was performed among 201 strains of *M. tuberculosis* retrieved from tuberculosis patients from 1961 to 1967 (15). These strains were retrieved from Danish-born patients who were suspected of being part of various chains of local transmission. Ninety-five came from case-patients living in Copenhagen, the capital city, and its surroundings, where most new tuberculosis cases were, and still are, found. The strains were freeze-dried in the 1960s

and recently recultured, and DNA fingerprinting was carried out (16,17). The strains were processed as previously described (3,16,17). The study was approved by the local medical ethics committees (No. 11-087/99) and the Danish Data Protection Agency (No. 2001-41-1018).

### Identification of Beijing Strains

Within the framework of the current European Union Concerted Action project (CA project), "New Generation Genetic Markers and Techniques for the Epidemiology and Control of Tuberculosis," a method of identifying the Beijing family of strains by using IS*6110* RFLP typing was defined, on the basis of comparison with 19 reference strains (https://hypocrates.rivm.nl/bnwww/index.html) (K. Kremer et al., unpub. data). Following the CA project suggested methodology, strains of *M. tuberculosis* with IS*6110* patterns with >80% similarity to any of these strains could be classified as Beijing family strains, whereas strains showing 75% to 80% similarity needed to be confirmed by spoligotyping. This procedure should give a sensitivity of >98% and specificity of 100% for recognizing Beijing family strains (compared with the standard criterion of spoligotyping) (K. Kremer et al., unpub. data). For this study, for all strains showing at least 75% similarity to any of the reference strains, spoligotyping was used to confirm that they were indeed Beijing strains. For statistical analysis, the p values were calculated by the chi-square test or Fisher exact test when expected values were small.

## Results

Among the strains from the 1960s, no Beijing family strains were identified. The results from the more recent patients are summarized in Table 1. In total, 96 Beijing strains were retrieved from different patients. The spoligo patterns of 95 of these strains had 9 spacers and 1 strain (from a patient from Vietnam) had 7 spacers of the spacers 35 to 43. Overall, 56% of the tuberculosis patients were born outside of Denmark, originating from 85 different countries. Among Danish-born patients, 1.0% had Beijing strains compared to 3.6% among foreign-born patients (Table 1). The highest prevalence of Beijing strains was among patients from Asia. By country of origin the prevalence of the Beijing strain varied: 25.0% (24/96) from Vietnam, 33.3% (12/36) from Thailand, 0% (0/44) from the Philippines, 9.7% (3/31) from India, 8.8% (3/34) from

Table 1. Proportion of tuberculosis patients with Beijing family strains[a]

| | Denmark-born N/N (%) | Non–Denmark-born N/N (%) | Total N/N (%) |
|---|---|---|---|
| All | 17/1,659 (1.0) | 79/2,183 (3.6) | 96/3,844 (2.5) |
| Male | 9/1,057 (0.85) | 49/1,163 (4.2) | 58/2,220 (2.6) |
| Female | 8/602 (1.3) | 30/1018 (3.0) | 38/1,620 (2.4) |
| Age group (y) | | | |
| < 25 | 2/118 (1.7) | 21/655 (3.2) | 23/773 (3.0) |
| 25–44 | 7/553 (1.3) | 48/1,159 (4.1) | 55/1,712 (3.2) |
| 45–64 | 4/522 (0.77) | 6/247 (2.4) | 10/769 (1.3) |
| 65+ | 4/466 (0.86) | 4/121 (3.3) | 8/587 (1.4) |
| Y | | | |
| 1992–93 | 4/335 (1.2) | 12/249 (4.8) | 16/584 (2.7) |
| 1994–95 | 2/371 (0.54) | 19/418 (4.6) | 21/789 (2.7) |
| 1996–97 | 2/330 (0.61) | 16/506 (3.2) | 18/836 (2.2) |
| 1998–99 | 4/316 (1.3) | 15/555 (2.7) | 19/871 (2.2) |
| 2000–01 | 5/307 (1.6) | 17/455 (3.7) | 22/764 (2.9) |
| Area of origin | | | |
| Western Europe | 17/1,659 (1.0) | 0/71 (0.0) | 17/1,730 (0.98) |
| Eastern Europe | | 0/174 (0.0) | |
| Indian subcontinent | | 8/290 (2.8) | |
| South East Asia | | 37/183 (20.2) | |
| East Asia and Pacific | | 3/10 (30.0) | |
| Middle East | | 6/211 (2.8) | |
| North Africa | | 1/38 (2.6) | |
| Sub–Saharan Africa | | 20/1,111 (1.8) | |
| Americas and Caribbean | | 0/16 (0.0) | |
| Previous TB | | | |
| No | 17/1550 (1.1) | 78/2164 (3.6) | 95/3,716 (2.6) |
| Yes | 0/109 (0.0) | 1/19 (5.3) | 1/128 (0.79) |
| Site of TB | | | |
| Pulmonary | 16/1,394 (1.2) | 56/1248 (4.5) | 72/2,642 (2.7) |
| Extrapulmonary | 1/263 (0.38) | 23/930 (2.2) | 24/1,193 (2.0) |

[a]Information on immigration status missing for three patients; on region of origin for 81; on age for 3; on sex for 4; and on site of tuberculosis (TB) for 9.

Sri Lanka, and 0% (0/220) from Pakistan. Beijing strains were also found in 1.7% of patients from Somalia (17/985) and in patients from the Middle East, including 7.5% (3/40) from Iraq, 10.5% (2/19) from Iran, and 3.9% (1/ 26) from Afghanistan. No Beijing strains were found in patients from Eastern Europe: most of these patients (149) were from the former Yugoslavia; 6 were from the former Soviet Union.

No evidence was noted of an increase in the prevalence of Beijing strains over time. Although no Beijing strains were found in the 1960s, this finding is not significantly different from the prevalence among Danish patients in the recent period ($p = 0.2$). No increase occurred over the period of the current study from 1992 to 2001 among Danish patients or those born outside of Denmark (Table 1). An apparent trend towards an increased proportion of Beijing strains in younger patients seen overall (Table 1) is attributable to the higher proportion of immigrants in younger age groups. Only one of the patients with the Beijing strain had known previous tuberculosis (a patient from Somalia). Beijing strains were less common in those without pulmonary involvement ($p = 0.007$, adjusted for immigration). HIV status was not available for these patients.

The results of drug resistance testing are shown in Table 2. Among Danish patients, but not among immigrants, the infections of those who had Beijing strains were more likely to be drug resistant. The results, after excluding those with known previous tuberculosis, were very similar (not shown). Although some of these associations were formally statistically significant, they are based on only two drug-resistant cases among 16 Danish-born patients with Beijing strains.

## Discussion

This population-based study found a low prevalence of Beijing strains and weak evidence of an association with drug resistance. The study includes an estimated 8% of all strains of *M. tuberculosis* IS*6110* RFLP typed worldwide from 1992 through 2001, of which 57% were retrieved from foreign-born patients from 85 different countries. Overall, only 2.5% of the patients had Beijing strains, and no evidence was found of an increase in their prevalence over time, even though Beijing strains have been found in Denmark for at least 10 years.

Recently, two studies analyzed the significance of *M. tuberculosis* transmission in Denmark due to immigration from a high incidence country and the persistent high incidence of tuberculosis among the immigrants in the years after arrival (3,18). These studies concluded that most (>75%) were infected before their arrival, that their latent infection was reactivated, and that nearly all of those who could have been infected after arrival (<23%) were most likely infected by a source from the country of origin (3). Therefore, in the present study we compared the observed prevalence with the prevalence in the country of origin. For example, 25% of patients from Vietnam had Beijing strains compared with 54% of patients in Hanoi and Ho Chi Minh City (8). However, the Vietnamese study included 563 samples from the late 1990s, whereas most Vietnamese-born immigrants arrived in Denmark during the early 1980s (19). This finding could indicate that Beijing strains have been emerging in Vietnam only since the early 1980s, which would fit with the higher prevalence of Beijing strains in persons of younger ages observed in the Vietnamese study. Regarding strains from patients born in Eastern Europe, none of the 174 patients had Beijing strains, compared with reports of 22% to 71% (4,20–22). However, the strains analyzed most were from patients from the former Yugoslavia, where the prevalence of Beijing strains is unknown. These patients arrived in Denmark during the 1990s. Our data suggest that the prevalence of Beijing strains was very low in this area, at least at that time. Few reports from Africa are available (23–26). In the present study, 17 (1.7%) of the 985 Somalia-born patients, nearly all of whom arrived in Denmark during the 1990s (18), had Beijing strains.

Table 2. Proportion of patients with drug-resistant strains

| | N | % Drug resistant (no. of patients with drug resistance)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Any drug | Isoniazid | Rifampicin | Streptomycin | Pyrazinamide | Ethambutol | MDR[b] |
| Danish | | | | | | | | |
| Beijing | 16 | 12.5 (2) | 12.5 (2) | 6.3 (1) | 12.5 (2) | 0.0 (0) | 6.3 (1) | 6.3 (1) |
| Other | 1,623 | 10.2 (165) | 3.1 (50) | 0.12 (2) | 3.6 (58) | 5.5 (89) | 0.0 (0) | 0.0 (0) |
| p | | 0.7 | 0.09 | 0.03 | 0.1 | 1.0 | 0.01 | 0.01 |
| Immigrants | | | | | | | | |
| Beijing | 78 | 20.5 (16) | 9.0 (7) | 0 (0) | 16.7 (13) | 1.3 (1) | 1.3 (1) | 0.0 (0) |
| Other | 2,086 | 17.2 (359) | 7.5 (157) | 0.72 (15) | 13.6 (284) | 1.3 (27) | 0.96 (20) | 0.58 (12) |
| p | | 0.4 | 0.7 | 1.0 | 0.4 | 1.0 | 0.5 | 1.0 |
| Overall | | | | | | | | |
| Beijing | 94 | 19.2 (18) | 9.6 (9) | 1.1 (1) | 16.0 (15) | 1.1 (1) | 2.1 (2) | 1.1 (1) |
| Other | 3,709 | 14.1 (524) | 5.6 (207) | 0.46 (17) | 9.2 (342) | 3.1 (116) | 0.54 (20) | 0.32 (12) |
| p | | 0.2 | 0.1 | 0.4 | 0.05 | 0.5 | 0.1 | 0.3 |

[a]Drug resistance data missing for 41 persons.
[b]MDR, multidrug resistant.

Among the remaining 126 patients, who were born in 24 other African countries, three additional Beijing strains were retrieved, from patients born in Zimbabwe, Kenya, and Angola. Beijing strains seem to be rare on the African continent, but local studies are needed. Immigrants are not a random sample, and some may have acquired tuberculosis en route.

This is one of the largest samples of strains of *M. tuberculosis* searched for Beijing strains. Although highly representative for the Danish population in the 1990s, and partly for the Danish-born population in the 1960s, the IS*6110* RFLP patterns found in the strains from the foreign-born patients may not be an accurate reflection of the distribution of patterns in their country of origin. Also, identified patterns are a mixture of "recent" *M. tuberculosis* transmission and reactivation of latent infections and thus also represent patterns circulating decades ago (16,17).

The low prevalence we found contrasts with some reports, but limited information is available from most areas of the world, making definite conclusions about the extent of spread of Beijing strains and their associations with drug resistance premature (4). Studies in which Beijing strains have been looked for but not found may not have been published. Recently two studies from Delhi and Bombay, India, reported very few Beijing family strains (27,28). Similarly, both in this study and in a previous study, the prevalence of Beijing strains in the Phillippines was found to be very low, 0% and 2%, respectively (29). These findings indicate that even in Asia prevalence may show great variation. More unbiased studies, even those that report negative findings, are needed. However, the body of typing data is increasing, thereby disclosing a growing part of the true tuberculosis picture.

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Dr. Lillebaek is a scientist in the International Reference Laboratory of Mycobacteriology, Statens Serum Institut, the National Institute for Prevention and Control of Infectious Diseases and Congenital Disorders, Copenhagen, Denmark. His research interests include infectious disease epidemiology, in particular, tuberculosis control and the molecular epidemiology of tuberculosis.

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Address for correspondence: Troels Lillebaek, Statens Serum Institut, International Reference Laboratory of Mycobacteriology, Artillerivej 5, DK-2300 Copenhagen S, Denmark; fax: +45 32 68 38 71; email: tll@ssi.dk

# *Trypanosoma cruzi* in Persons without Serologic Evidence of Disease, Argentina

**Oscar A. Salomone,* Ana L. Basquiera,* Adela Sembaj,† Ana M. Aguerri,† María E. Reyes,‡ Mirtha Omelianuk,* Ruth A. Fernández,† Julio Enders,† Atilio Palma,† José Moreno Barral,† and Roberto J. Madoery***

Current diagnosis of chronic Chagas disease relies on serologic detection of specific immunoglobulin G against *Trypanosoma cruzi*. However, the presence of parasites detected by polymerase chain reaction (PCR) in patients without positive conventional serologic testing has been observed. We determined the prevalence and clinical characteristics of persons with seronegative results and *T. cruzi* DNA detected by PCR in a population at high risk for chronic American trypanosomiasis. We studied a total of 194 persons from two different populations: 110 patients were recruited from an urban cardiology clinic, and 84 persons were citizens from a highly disease-endemic area. Eighty (41%) of persons had negative serologic findings; 12 (15%) had a positive PCR. Three patients with negative serologic findings and positive PCR results had clinical signs and symptoms that suggested Chagas cardiomyopathy. This finding challenges the current recommendations for Chagas disease diagnosis, therapy, and blood transfusion policies.

American trypanosomiasis or Chagas disease is usually asymptomatic; for this reason, its diagnosis is mainly based on laboratory tests. During the indeterminate and chronic clinical periods, detection of immunoglobulin (Ig) G against *Trypanosoma cruzi* by more than two different serologic tests is the standard for diagnosis (1). Moreover, serodiagnosis is used for epidemiologic surveillance, to evaluate efficacy of therapy, and for routine testing in blood banks.

Conversely, direct identification of *T. cruzi* is the main tool for diagnosis during the acute phase of Chagas disease. During the other phases of the disease, detection of the parasites is rare because of low levels of parasitemia. However, the development of polymerase chain reaction (PCR) has allowed detection of *T. cruzi* in a higher number of patients with chronic disease. In this stage, the prevalence of circulating parasites varies from 21% to 100% by using PCR, and this variability may be associated with episodes of reinfection (2–4). Previous reports have focused on the high sensitivity of PCR test when compared to serologic findings, xenodiagnosis, or blood culture. Nonetheless, in some of these investigations a discordant finding has been observed; parasitemias have been detected by PCR from serum samples of seronegative persons (5–9). Although the parasite has been directly observed in blood of seronegative patients (5), this problem has been largely ignored in the clinical setting. Parasitemias in patients with negative serologic findings could represent a sanitary problem since most diagnostic and therapeutic recommendations rely on a serologic test.

We conducted a cross-sectional study in two populations at high risk for Chagas disease to evaluate the prevalence of positive *T. cruzi* PCR results in seronegative persons. We describe the results of that study as well as the clinical characteristics of a subgroup of patients.

## Patients and Methods

### Population and Protocol Study

We studied 194 persons from two populations. We included an urban population of 110 consecutive patients who attended the Cardiology Clinic of the Hospital Privado de Córdoba, Argentina, with epidemiologic or clinical suspicion of Chagas disease. All the patients were permanent residents of the city of Córdoba during the last 10 years. Córdoba is considered a low Chagas-endemic area. The other group consisted of 84 persons from La Posta, a small rural village of 384 persons located in a northern rural area of the province of Córdoba. This area is highly endemic for Chagas disease. All residents of this area ≥14 years of age were invited to participate in the study through informative workshops conducted by spe-

*Hospital Privado Centro Médico de Córdoba, Córdoba, Argentina; †Universidad Nacional de Córdoba, Córdoba, Argentina; and ‡Ministerio de Salud de Córdoba, Córdoba, Argentina

cially trained sanitary agents. The study protocol was designed according to Helsinki's Declaration, and informed consent was obtained for all patients.

All patients completed an epidemiologic and clinical questionnaire and had a physical examination. Also, both urban and rural participants had a 12-lead electrocardiogram and a transthoracic echocardiogram.

### Serologic Tests

Three serologic assays for all case-patients were performed to detect chronic *T. cruzi* infection: indirect immunofluorescence assay (IFA, positive ≥1:32 dilution; Biocientífica, Buenos Aires, Argentina), hemagglutination inhibition assay (positive ≥1:28 dilution, Biochagas, Biocientífica, Buenos Aires, Argentina), and enzyme-linked immunosorbent assay (ELISA, Abbott Labs, Abbott Park, Illinois). Chronic Chagas disease was defined as the presence of ≥2 positive serologic determinations (1). Also, anti-cruzipain antibodies were detected by ELISA as previously described (10,11).

### PCR for Identification of *Trypanosoma cruzi*

Peripheral blood samples were drawn from each study participant for PCR detection of *T. cruzi,* as previously described (12,13). Four milliliters of blood was transferred to guanidine-EDTA containing tubes until DNA extraction. We collected 600 mL of blood to separate DNA by using conventional technique of fenol: chloroform: isoamyl alcohol and then ethanol precipitation. Finally, the solution was suspended in free-endonuclease sterile water. DNA amplification was carried out in 50 mL of a mixture containing 10 mM 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each deoxinucleoside triphosphate, 1.25 U Taq polymerase (Perkin Elmer Cetus Corp, Norwalk, CT), and 1 mM of each primer. We amplified a sequence of 220 bp, which corresponds to a family of E13 genes with high repetition in the genome of *T. cruzi*; the sequence of the primers used was: O1, 5′-TGGCTTGGAGGAGTTATTGT-3′; O2, 5′-AGGAGTGACGGTTGATCAGT-3′ (12). The reaction was initiated with 10 min of denaturalization at 94°C and 30 cycles of amplification, each consisting of 1 min at 94°C, 1 min at 55°C, and 2 min at 63°C in a Perkin-Elmer-Cetus terminal cycler. We analyzed the PCR product in a 1.6% agarose gel. In all samples, DNA from cultivated *T. cruzi* epimastigotes of Tulahuen strain was used as positive control. Negative control consisted of a specimen without DNA. Also, 330-bp fragment of the β-actin gene (Promega, Madison, WI) was amplified with the same procedure as E13 fragment to check DNA quality and to show amplification inhibitors.

### Statistical Analysis

Data are presented as mean ±SD or as number and percentage of cases. We used chi-square test to compare categorical variables between groups. A value of $p < 0.05$ was considered significant.

## Results

Characteristics of both urban and rural populations are shown in Table 1. Results of serologic testing for 76 (69%) and 38 (44%) persons from urban and rural populations, respectively, were positive for *T. cruzi* infection. Globally, 80 (41%) persons did not fulfill criteria of serologic diagnosis of Chagas disease (in all cases, IFA test was negative). In eight of nine rural seronegative patients, anti-cruzipain antibodies were investigated with negative results.

*T. cruzi* was detected by PCR amplification of a nuclear DNA fragment by using the O1/O2 primers (see Materials and Methods). This reaction has been previously demonstrated to be highly specific to detect *T. cruzi* in blood samples (12). Parasitemia by PCR assay was detected in 34 (17%) of 194 persons and was more frequently found in rural than in urban populations (20 and 14 positive persons, respectively; $p = 0.05$) (Figure). When only the seronegative population was considered, PCR was positive in 12 (15%) persons (3 and 9 from urban and rural population, respectively; $p = 0.36$). Clinical characteristics of these patients are shown in Table 2. Only one patient (from the urban group) had a previous positive Machado Guerreiro test. Two of three urban patients were born in a highly disease-endemic area. Disease in all of these urban patients was controlled a year after recruitment, and subsequent serologic testing was negative. Of the rural case-patients (born and living in La Posta), none reported previous positive serologic findings. Electrocardiogram and echocardiogram were performed for four patients from a rural area (Table 2).

Table 1. Demographic and laboratory characteristics of study participants[a]

| Variable | Total (n = 194) | Urban population (n = 110) | Rural population (n = 84) |
|---|---|---|---|
| Age, mean ± SD (y) | 52 ±14 | 56 ±14 | 48 ±15 |
| Male (%) | 36 | 37 | 33 |
| Negative serologic finding, n (%) | 80 (41) | 34 (31) | 46 (54) |
| Positive PCR assay, n (%) | 34 (17) | 14 (13) | 20 (24) |
| Positive PCR assay and negative serologic findings, n (%) | 12 (6) | 3 (9) | 9 (20) |

[a]PCR, polymerase chain reaction.

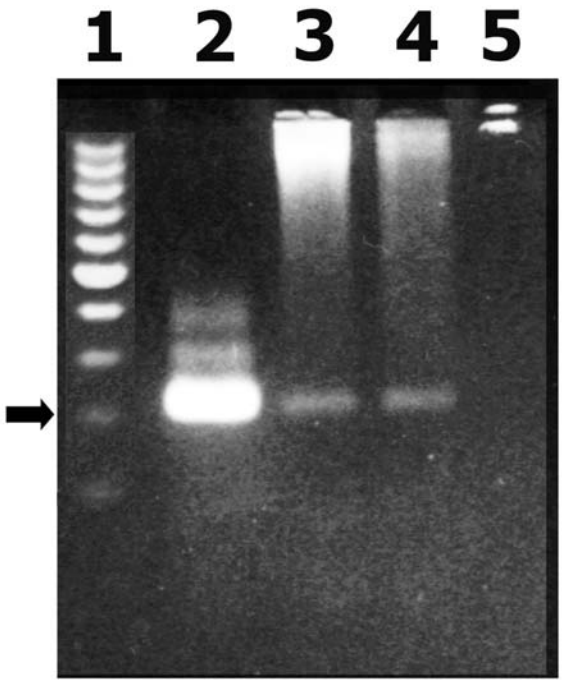


Figure. Gel electrophoresis analysis of a polymerase chain reaction (PCR) product corresponding to a highly repetitive 220-bp *Trypanosoma cruzi* nuclear fragment. 1: molecular weight standards, 2: *T. cruzi* nuclear 220-bp PCR product, 3 and 4: PCR product from patients blood, 5: PCR negative control (arrows correspond to 220 bp).

## Discussion

When *T. cruzi* infects a mammal, several immunologic reactions occur that eliminate the parasite. First, a cellular immune response attempts to isolate the microorganism and avoid its wide spread. Simultaneously, a humoral response occurs, with IgM antibodies first and IgG antibodies 2–3 weeks later. However, because of the lack of efficacy of these mechanisms, the parasite persists in low-density tissues and in turn, triggers an inflammatory response, resulting in tissue damage during the chronic period of the disease (14). Parasites are rarely isolated from blood or tissue from chronically infected patients, and the diagnosis is based on serologic analysis.

In our study, we observed that persons with positive *T. cruzi* in blood and negative serologic findings could be detected in a population with high epidemiologic risk. This observation has been previously reported in Wincker et al. (5), who studied PCR technique using serum samples from 45 Bolivian children. They found two positive PCR results in 17 seronegative children, and in one of them, parasites were seen on direct blood examination. These authors also reported a patient with the same infectious condition in 268 children with high epidemiologic risk for Chagas disease (6). In Brazil, Avila et al. (7) observed three discordant cases, one of which had typical findings of myocardial damage. Similarly, Castro et al. (9) detected 3 persons with positive PCR results among 9 seronegative controls, and Gomez et al. (8), reported 10 positive PCR results in seronegative patients of 110 residents of a highly disease-endemic region.

Several arguments have been proposed to explain this controversial situation. Recent infection that has not yet been recognized by the immune system of persons highly exposed to vectorial infections is one possible explanation. However, acute infection is not a frequent event in our study population because of age and because urban population is infrequently exposed to vectorial reinfection. Alternatively, one could speculate that positive samples could have been contaminated with DNA, but this theory has been disregarded by many authors (8,9). We repeated serologic and PCR assays three times for each patient with two different operators, and we obtained the same results. Finally, *T. cruzi* may chronically infect some patients but a humoral response may not develop or be detected by conventional serologic testing. Addressing this point, Castro et al. (9) observed that 80% of seronegative but positive PCR patients had lytic antibodies against *T. cruzi* by a complement-mediated lysis test (CoML). Similarly, Leguizamón et al. (15) have reported patients who were seropositive for Chagas disease only by inhibition transamidase assay but negative with conventional serologic testing. To test this hypothesis, we searched for anti-cruzipain antibodies in eight rural patients, but all of them were negative.

Otherwise, independently of its cause, considering the clinical and diagnostic consequences of this phenomenon is necessary. In our study, at least 3 of the 12 patients with high epidemiologic risk for Chagas disease had signs of cardiac compromise. The consensus is that the detection of DNA constitutes real proof of parasites. DNA detected in blood is originated from extracellular parasites recently liberated or destroyed. According to this theory, Tarleton and Zhang observed that after injection of high doses of kinetoplastic DNA (kDNA) of *T. cruzi* in muscle, kDNA is detected in blood 2 days later (16). However, since the parasite is infective as tripomastigotes but not as a portion of DNA, we cannot be sure that DNA detected by PCR in blood is a reliable surrogate of infecting *T. cruzi* forms. One alternative is the possibility that fragments of amastigotes (the tissue-infecting forms) reach the bloodstream after interacting with the immune system. Even though this consideration may be found relevant in the design of transfusion policies, no reliable information currently exists, and the potential for these persons to transmit the disease is still uncertain.

Comparing our results with a population without any risk for Chagas disease to determine the predictive value of PCR among seronegative persons would be interesting. However, the accuracy of PCR has been studied in depth

Table 2. Epidemiologic and clinical characteristics of 12 patients with negative serologic findings and positive PCR for *T. cruzi* in blood

| Patient | Age (y) | Sex | Previous positive serologic test | ECG | Echocardiogram | |
|---|---|---|---|---|---|---|
| | | | | | LVDd (mm) | LVEF (%) |
| Urban 1 | 56 | F | Present | Normal | 44 | 64 |
| Urban 2 | 66 | F | Absent | RBBB | 42 | 65 |
| Urban 3 | 58 | M | Absent | RBBB + LAFB | 45 | 60 |
| Rural 1 | 35 | F | Absent | ND | ND | ND |
| Rural 2 | 17 | F | Absent | ND | ND | ND |
| Rural 3 | 15 | F | Absent | ND | ND | ND |
| Rural 4 | 47 | F | Absent | Normal | 45 | 50 |
| Rural 5 | 59 | M | Absent | ND | ND | ND |
| Rural 6 | 22 | F | Absent | ND | ND | ND |
| Rural 7 | 24 | M | Absent | IRBBB | 50 | 45 |
| Rural 8 | 68 | M | Absent | Normal | 39 | 56 |
| Rural 9 | 43 | F | Absent | Normal | 46 | 64 |

[a]PCR, polymerase chain reaction; F, female; M, male; ECG, electrocardiogram; RBBB, right bundle branch block; IRBBB, incomplete right bundle branch block; LAFB, left anterior fascicular block; LVDd, left ventricular diameter in diastole; LVEF, left ventricular ejection fraction; ND, not determinate.

(2–4,12,17). While previous reports of PCR in Chagas disease used a sequence of kDNA of *T. cruzi* to detect the parasite, we used nuclear DNA that has been also validated (12,13). Currently, we have not carried out a systematic comparison of the PCR sensitivity for different *T. cruzi* sequences. We considered determining which of the different PCR systems cited in the literature is the more sensitive and accurate for detection of parasitemia in blood specimens of patients with chronic Chagas disease.

In summary, we found a prevalence of 15% of *T. cruzi* DNA for American trypanosomiasis in a seronegative population living in Chagas-endemic regions. We also observed that some of these persons had cardiac abnormalities suggestive of Chagas cardiomyopathy. Experts should consider these finding when making diagnostic, therapeutic, and transfusion recommendations.

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Dr. Salomone is a staff member of the Cardiology Department of Hospital Privado de Córdoba. His research interests include pathogenesis, diagnosis, and treatment of Chagas disease, and he is currently working on his doctoral thesis on Chagas disease.

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Address for correspondence: Oscar A. Salomone, Hospital Privado Centro Médico de Córdoba, Naciones Unidas 346 (5016) Córdoba, Argentina; fax: (54-351) 468-8865; email: nobaks@onenet.com.ar

# Risk Factors for Norovirus, Sapporo-like Virus, and Group A Rotavirus Gastroenteritis

**Matty A.S. de Wit,*[1] Marion P.G. Koopmans,* and Yvonne T.H.P. van Duynhoven***

Viral pathogens are the most common causes of gastroenteritis in the community. To identify modes of transmission and opportunities for prevention, a case-control study was conducted and risk factors for gastroenteritis attributable to norovirus (NV), Sapporo-like virus (SLV), and rotavirus were studied. For NV gastroenteritis, having a household member with gastroenteritis, contact with a person with gastroenteritis outside the household, and poor food-handling hygiene were associated with illness (population attributable risk fractions [PAR] of 17%, 56%, and 47%, respectively). For SLV gastroenteritis, contact with a person with gastroenteritis outside the household was associated with a higher risk (PAR 60%). For rotavirus gastroenteritis, contact with a person with gastroenteritis outside the household and food-handling hygiene were associated with a higher risk (PAR 86% and 46%, respectively). Transmission of these viral pathogens occurs primarily from person to person. However, for NV gastroenteritis, foodborne transmission seems to play an important role.

Recent studies in the Netherlands and other countries have shown that viral infections, especially noroviruses (NV), are the most frequent cause of gastroenteritis in the community, both outbreak-related and endemic (1–8). The overall incidence of gastroenteritis in the Netherlands was estimated at 283 per 1,000 persons per year in a community-based study in 1999 (3). NV was detected in 11% of cases, Sapporo-like viruses (SLV) in 2%, and rotavirus group A in 4%. The incidence at the general-practice level from 1996 to 1999 was estimated at 14 per 1,000 person-years; 5% was attributed to NV, 2% to SLV, and 5% to rotavirus (2,9). For rotavirus, preventive measures currently focus on developing vaccines to reduce hospitalizations in cases where the illness is complicated by dehydration. For caliciviruses, in spite of their high incidence, little effort has gone into prevention, and little is known about preventable routes of infection. Although possible transmission routes, such as food products or water supplies (10,11), were identified in outbreaks, sources in endemic cases are difficult to detect. Therefore, we conducted a case-control study to identify risk factors that could provide leads for preventing endemic cases of viral gastroenteritis attributable to caliciviruses and rotavirus.

## Methods

A community-based prospective cohort study with a nested case-control study was undertaken in the Netherlands in 1999 (3). The cohort was followed to estimate the incidence of gastroenteritis. The nested case-control study was used to identify risk factors and determine etiology. The study was performed in cooperation with the sentinel general practice network of the Netherlands Institute of Primary Health Care. The cohort consisted of an age-stratified sample of persons registered at general practices in this network. Cases identified in the community-cohort with gastroenteritis were included in the case-control study, and a matched control was selected from the cohort members without gastroenteritis at that time. Case-patients and controls were matched by age, degree of urbanization, region, and date of inclusion. At the start of follow-up, all persons in the cohort completed a questionnaire on demographic characteristics and long-term risk factors (such as food-handling practices and presence of animals). Case-patients and controls included in the case-control study completed a questionnaire addressing short-term risk factors in the 7-day period before onset of symptoms and submitted stool samples. Case-patients submitted four stool samples (on days 1, 8, 14, and 21 of the episode), and controls submitted two stool samples (on days 1 and 8 from inclusion as a control). Samples were tested for NV and SLV by reverse transcription-polymerase chain reaction and for rotavirus group A by enzyme-linked immunosorbent assay, as described (3,12–14).

*National Institute of Public Health and the Environment, Bilthoven, the Netherlands

[1]Current address of first author: Municipal Health Service, Department of Epidemiology, Documentation and Health Promotion, Nieuwe Achtergracht 100, 1018 WT Amsterdam, the Netherlands; email: mdwit@gggd.amsterdam.nl

### Potential Risk Factors

Potential risk factors we studied were chronic gastrointestinal symptoms, being breastfed, having animals in the household (both pets and farm animals), food-handling hygiene index, method of keeping and heating up leftover food, presence of household equipment (blender, dishwasher, microwave, freezer), child in diapers in household, participant or other child in household attending a daycare center or primary school, size of household, being pregnant, being vegetarian, nationality, country of birth of participant and parents, being employed, type of house, income, educational level, age, and sex. The following factors were studied in the week before onset of illness or before inclusion as a control: contact with others with gastroenteritis (in and outside the household); swimming or other water-related sports; foreign travel; use of antimicrobial drugs, consumption of (raw or well-done) chicken, pork, beef, organ meat, meat in dough, fish, crab, shrimp, oysters, mussels, raw vegetables, salad, fruits, dried fruits, rice, raw milk, ice cream, soft cheeses, runny eggs, raw eggs, take-away fast-food, take-away bread rolls, take-away kebab, take-away Chinese food, meal services, food from canteen, food from reception, food from barbecue, eating out in a restaurant, and contact with farm animals (with or without diarrhea).

### Statistical Analyses

All gastroenteritis case-patients who tested positive in the first or second stool samples and their matched controls were included in the analyses. Gastroenteritis was defined as one of the following: three or more loose stools in 24 hours; three or more vomiting episodes in 24 hours; diarrhea with at least two additional symptoms; or vomiting with at least two additional symptoms. Additional symptoms were abdominal pain, abdominal cramps, nausea, blood in stool, mucus in stool, fever, diarrhea, or vomiting.

Univariate analyses were completed by using McNemar and Bowkers test for symmetry for categorical variables and paired t tests and Wilcoxon signed rank test for continuous variables. A conditional logistic regression model was used to study the independent effects of risk factors with an association in the univariate analyses with a p value of $<0.10$. Selection of variables in the model was backwards manually, based on the log-likelihood ratio; a significance level of 0.05 was used.

All risk factors in the questionnaire were studied to have the possibility to generate hypotheses on transmission, in addition to confirming and clarifying existing theories. Since the specific variables on food handling in the questionnaires were mainly focused on possible risk factors for bacterial gastroenteritis, they were used as indicators of food-handling hygiene in these analyses. An index was made for food-handling hygiene on the basis of several indicator variables. Two different scores were developed: a basic score, calculated by adding up all factors and weighing them equally, and an optimized score, which used the $\beta$ from a logistic model as the weight for each factor. This logistic model was fit on NV gastroenteritis as an outcome because this was the largest group. The following variables were included as indicators (factors marked with an asterisk were independent indicators in the optimized score): frequency of shopping, *checking the appearance of product in shop, checking the packaging for damage in shop, following the storage instructions, checking the expiration date, *duration of keeping eggs, *use of same cutting board for raw meat and other products, *washing of cutting board between use for raw meat and other products, and frequency of changing dish brush, *scourer, and dishcloth.

The effect of food-handling hygiene includes both the effect of poor food-handling hygiene in the household favoring indirect person-to-person transmission and foodborne infection by introduction of contaminated food into the household. To estimate the second effect separately, we estimated the proportion preventable by hygienic food handling among those not in contact with other persons with gastroenteritis in the last week. This estimate was made by calculating the incidence attributable to food-handling hygiene among those not exposed to other persons with gastroenteritis and dividing it by the total incidence of virus-specific gastroenteritis. We assumed that all persons who reportedly had contact with a person with gastroenteritis were infected by that person.

Because age was likely to interact with all variables, we constructed a separate model for the age groups $<5$ years and $\geq 5$ years. This stratification was possible for NV only because not enough adults were infected with rotavirus and SLV to make the analysis.

Population-attributable risk fractions (PAR) were calculated on the basis of multivariate odds ratios (OR) by estimating the incidence attributable to the risk factor and dividing it by the total incidence of virus-specific gastroenteritis. The total incidence of virus-specific gastroenteritis was calculated by multiplying the proportion positive and the overall incidence of gastroenteritis in the cohort. The incidence attributable to the risk factor was calculated as the total virus-specific incidence minus the estimated incidence if the risk factor was absent, which was estimated by weighing the cases according to their exposure status. Exposed cases were weighed as 1/OR of exposure, nonexposed cases as 1. All incidence estimates were standardized by age and cohort. Data from the case-control study were extrapolated to the entire cohort.

## Results

### Norovirus (NV)

In total, 152 case-patients were positive for NV—57 in both stool samples, 57 only in the first sample, and 38 only in the second sample. Of the matched controls, seven were positive for NV but did not have gastroenteritis. The median age of case-patients was 2 years (age distribution: <1 year: 47 [31%]; 1–4 years: 60 [39%]; 5–9 years: 25 [16%]; 10–59 years: 12 [8%]; ≥60 years: 8 [5%]).

NV gastroenteritis was independently associated with food-handling hygiene, having more than one household member with gastroenteritis (hereafter referred to as household gastroenteritis contact), and having contact with a person with gastroenteritis outside the household (hereafter referred to as outside gastroenteritis contact) in the week before onset of symptoms (Table 1, Figure 1). For the risk factor of household gastroenteritis contact, risk was slightly higher if the household member was a child rather than an adult (5.2 [95% confidence intervals (CI) 1.8 to 15.3] vs. 4.4 [95% CI 2.0 to 9.6]). Risks were comparable if the household member had diarrhea or vomiting. Because of the strong correlation of all variables on household contacts, only the number of household gastroenteritis contacts was included in the model. The contact of cases with symptomatic persons outside the household had taken place in the house of friends or family (31%), a daycare center (19%), school (18%), home (10%), or other places and work (22%). Cases and controls did not differ significantly. The association of attendance at daycare and primary school with NV gastroenteritis in the univariate analysis was no longer observed after correction for household gastroenteritis contact, especially if the sick household member was a child.

Food-handling hygiene and outside gastroenteritis contact were the factors with the highest impact, as measured by PAR (Tables 1 and 2). PAR for all significant risk factors combined was 80%. PAR for outside gastroenteritis contact and household gastroenteritis contact combined accounted for 63% of cases. PAR for the two factors representing transmission in the household combined (hygiene and household gastroenteritis contact) was 56%. This figure is much lower than the sum of both PARs.

For persons ≥5 years of age, the effect of household gastroenteritis contact was reduced (OR = 1.1, PAR = 4%) when controlling for food-handling hygiene (Table 2). Both factors (food-handling hygiene and household gastroenteritis contact) combined showed a similar PAR in both age groups (60% vs. 65%). Use of an optimized food-handling hygiene score for NV gastroenteritis resulted in a higher estimate of PAR for food-handling hygiene (60%).

We estimated the effect of contaminated food's entering the household, separate from transmission from symptomatic contact persons through food to the patient, as

Table 1. Risk factors for NV gastroenteritis, prevalence in cases and controls (152 pairs), and univariate and multivariate odds ratios using logistic regression and population-attributable risk fractions[a]

| | Cases | Controls | | | | | |
|---|---|---|---|---|---|---|---|
| NV gastroenteritis | n (%) | n (%) | OR uni | 95% CI | OR multi | 95% CI | PAR (%) |
| Food-handling hygiene[b] | | | 1.3 | 1.0 to 1.5 | 1.3 | 1.0 to 1.7 | 47 |
| Educational level | | | | | n.i. | | |
| Low | 21 (14.3) | 16 (10.9) | 1.9 | 0.9 to 4.0 | | | |
| Intermediate | 58 (39.5) | 80 (54.4) | 1.0 | - | | | |
| High | 68 (46.3) | 51 (34.7) | 2.2 | 1.2 to 3.9 | | | |
| Participant to daycare center | 47 (30.9) | 37 (24.7) | 1.7 | 0.9 to 3.3 | n.i. | | |
| Household member to daycare center | 34 (23.5) | 21 (14.5) | 2.0 | 1.0 to 3.9 | n.i. | | |
| Household member to primary school | 62 (42.8) | 48 (33.1) | 1.6 | 1.0 to 2.7 | n.i. | | |
| Pets in household | 85 (56.3) | 102 (67.6) | 0.6 | 0.4 to 1.0 | n.i. | | |
| Cat as pet | 46 (30.5) | 61 (40.4) | 0.6 | 0.4 to 1.0 | n.i. | | |
| No. of household members with gastroenteritis[c] | | | | | | | 17 |
| None | 73 (48.3) | 130 (85.8) | 1.0 | - | 1.0 | - | |
| 1 | 39 (25.8) | 15 (10.0) | 3.7 | 1.7 to 8.0 | 1.2 | 0.3 to 4.2 | |
| >1 | 39 (25.8) | 6 (4.2) | 13.1 | 3.9 to 34.7 | 10.9 | 2.0 to 60.5 | |
| Contact with persons outside household with gastroenteritis[c] | | | | | | | 56 |
| No | 50 (32.9) | 101 (66.5) | 1.0 | - | 1.0 | - | |
| Yes | 57 (37.5) | 8 (5.3) | 11.4 | 4.7 to 27.3 | 12.7 | 3.1 to 51.8 | |
| Do not know | 45 (29.6) | 43 (28.3) | 1.9 | 1.1 to 3.4 | 2.5 | 1.0 to 6.5 | |
| Consumption of fish[c] | 46 (34.6) | 32 (24.1) | 1.8 | 1.0 to 3.2 | n.i. | | |
| Consumption of barbecued food[c] | 1 (1.5) | 9 (6.6) | 0.2 | 0.05 to 1.0 | n.i. | | |

[a]NV, norovirus; OR, odds ratio; PAR, population-attributable risk fraction; uni, univariate; multi, multivariate; CI, confidence interval; n.i, not in final model; -, not applicable.
[b]Basic score (not optimized), higher score indicates less hygienic practices, OR for increase of 1.
[c]In the week before onset of symptoms (case-patients), inclusion in study (control).

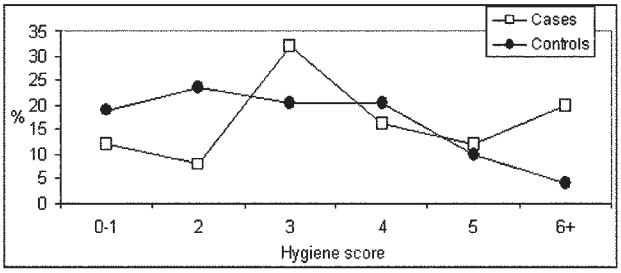


Figure 1. Distribution of basic food-handling hygiene score in norovirus gastroenteritis cases (n = 152) and controls (n = 152). (A higher score indicates less hygienic practices.)

explained in Methods. (We assumed that all case-patients who had contact with a person with gastroenteritis in the week before onset of symptoms were infected by these contacts.) Thirty-four pairs remained for the calculation of OR. For food-handling hygiene, OR was slightly higher for those not having contact with someone with gastroenteritis (1.4, 95% CI 0.8 to 2.2). The estimate of PAR for contaminated food's entering the household was 12% of all NV gastroenteritis cases and 16% when the optimized score was used.

### Sapporo-like Viruses

In total, 48 cases were positive for SLV—21 in both samples, 22 only in the first sample, and 5 only in the second sample. Of the matched controls, two were positive for SLV but did not have gastroenteritis. The median age of SLV gastroenteritis case-patients was 1 year (age-distribution: <1 year: 19 [40%]; 1–4 years: 21 [44%]; 5–9 years: 4 [8%]; 10–68 years: 4 [8%]).

Outside gastroenteritis contact was the only independent risk factor for SLV gastroenteritis (Table 3). The location of contacts of case-patients and controls did not differ significantly. For case-patients, 29% of contacts took place at daycare, 21% at homes of friends or family, 14% at home, and 7% at school.

### Rotavirus

In total, 54 cases were positive for rotavirus—11 in both samples, 41 only in the first, and 2 only in the second. None of the matched controls was positive for rotavirus. The median age of rotavirus gastroenteritis case-patients was <1 year (age distribution of cases: <1 year: 28 [52%]; 1–4 years: 18 [33%]; 5–9 years: 3 [6%]; 10–72 years: 5 [9%]).

Rotavirus gastroenteritis was independently associated with outside gastroenteritis contact and with food-handling hygiene (Table 4 and Figure 2). A strong independent negative association was found with presence of a blender in the household. By univariate analysis, a high education level was a risk factor for rotavirus gastroenteritis, as was a household gastroenteritis contact. The risk was higher if the household gastroenteritis contact was a child (OR 5.8, 95% CI 1.3 to 25.6) than if the contact was an adult (OR 4.0, 95% CI 0.5 to 38.2). The association of a household gastroenteritis contact disappeared after correction for outside gastroenteritis contact. The association with educational level disappeared after correction for food-handling hygiene. Locations of outside gastroenteritis contacts did not differ significantly between cases and controls. For cases, 40% of contacts took place at homes of friends or family, 30% at daycare centers, and 20% at home. PAR for outside gastroenteritis contact was 86%; for food-handling hygiene, PAR was 46%. PAR for both factors combined was 92%. Use of the optimized food-handling hygiene score resulted in a PAR for food-handling hygiene of 64% and PAR for all factors combined of 96%.

We estimated the effect of contaminated food's entering the household in the same way as we did for NV gastroenteritis. The case-patients in 10 pairs had not had any con-

Table 2. Risk factors for NV gastroenteritis in persons <1 year to 4 years of age (n = 105 pairs) and persons ≥5 years of age (n = 46 pairs), univariate and multivariate odds ratios[a,b]

| | <1 y to 4 y (n = 105 pairs) | | | | | ≥5 y (n = 46 pairs) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Risk Factor | OR uni | 95% CI | OR multi | 95% CI | PAR (%) | OR uni | 95% CI. | OR multi | 95% CI | PAR (%) |
| Food-handling hygiene | 1.2 | 0.9 to 1.5 | 1.2 | 0.9 to 1.7 | 46 | 1.3 | 0.9 to 1.9 | 1.3 | 0.8 to 2.2 | 63 |
| Household members with gastroenteritis | | | | | 27 | | | | | 4 |
| Yes | 4.4 | 2.2 to 9.2 | 2.7 | 0.8 to 8.9 | | 15.0 | 2.0 to 113.6 | 1.1 | 0.1 to 15.9 | |
| No | 1.0 | - | 1.0 | - | | 1.0 | - | 1.0 | - | |
| Contact with persons outside household with gastroenteritis | | | | | 51 | | | | | 60 |
| No | 1.0 | - | 1.0 | - | | 1.0 | - | 1.0 | - | |
| Yes | 17.7 | 5.1 to 61.1 | 10.9 | 2.2 to 54.6 | | 5.9 | 1.7 to 20.1 | 12.1 | 1.0 to 147.3 | |
| Do not know | 2.4 | 1.2 to 4.7 | 2.7 | 0.9 to 7.8 | | 0.8 | 0.2 to 3.0 | 1.8 | 0.2 to 15.3 | |

[a]Using logistic regression, and population-attributable risk fraction (PAR).
[b]NV, norovirus; OR, odds ratio; PAR, population-attributable risk fractions; uni, univariate; multi, multivariate; CI, confidence intervals; -, not applicable.

Table 3. Risk factors for SLV gastroenteritis, prevalence in cases and controls (48 pairs), univariate and multivariate odds ratios using logistic regression and PAR[a]

| | Cases | Controls | | | | | |
|---|---|---|---|---|---|---|---|
| SLV gastroenteritis | N (%) | N (%) | OR uni | 95% CI | OR multi | 95% CI | PAR (%) |
| Household member with gastroenteritis[b] | 19 (39.6) | 10 (21.3) | 2.8 | 1.0 to 7.8 | n.i. | | |
| Contact with person outside household with gastroenteritis[b] | | | | | | | 60 |
| No | 1 (25.0) | 28 (58.3) | 1.0 | - | 1.0 | - | |
| Yes | 14 (29.2) | 8 (16.7) | 4.4 | 1.3 to 14.9 | 4.4 | 1.3 to 14.9 | |
| Do not know | 22 (45.8) | 12 (25.0) | 4.1 | 1.4 to 11.6 | 4.1 | 1.4 to 11.6 | |

[a]SLV, Sapporo-like virus; PAR, population-attributable risk fraction; OR, odds ratio; uni, univariate; multi, multivariate; CI, confidence interval; n.i., not in final model.
[b]In week before onset of symptoms.

tact with a person with gastroenteritis. In this group, OR for food-handling hygiene was 1.8 (95% CI 0.8 to 3.9), which is higher than in the total group of rotavirus cases. The estimate of PAR for contaminated food's entering the household, based on these assumptions, was 4% of all rotavirus gastroenteritis cases.

## Discussion

To our knowledge, this study is the first to describe risk factors for the three main viral pathogens causing gastroenteritis and to estimate the effect of these risk factors in the population. The main risk factor for NV, SLV, and rotavirus gastroenteritis was contact with persons with gastroenteritis, supporting the hypothesis that these viruses are mainly transmitted from person to person (13,15). The high PARs indicate that most of these infections can indeed be prevented by stopping the spread from symptomatic persons to others.

Food-handling hygiene in the household was also strongly associated with risk for NV gastroenteritis and with a high PAR. This association indicates that in a household setting these viruses do not necessarily transmit directly from one person to another but by means of food. Hygienic food-handling procedures can therefore further prevent the infection spreading from one person to another (16).

The impact of food-handling hygiene can be partly explained by food contamination that occurs when a sick household member prepares meals. However, food contaminated at an earlier step in the food chain may also be a source. On the basis of our data, an estimated 12%-16% of NV gastroenteritis and 4% of rotavirus gastroenteritis cases are caused by introduction of contaminated food or water. This figure may be an overestimate, if infection through the shedding of other asymptomatic persons plays a major role, or if the knowledge of respondents about illness in their contacts is limited. Alternatively, the proportion of NV infections attributable to foodborne transmission might be an underestimate since we assumed that, if contact with symptomatic others had taken place, such contact was always the cause of illness. The 40% of NV infections that were foodborne, as presented by Mead (17) on the basis of NV outbreak surveillance, is higher than our 12%-16% estimate related to contaminated food's entering the household and comparable with the 47% related to food hygiene. Mead's estimate was extrapolated to a community incidence from preliminary NV data from our community study (8,17). Clearly, the precise number

Table 4. Risk factors for rotavirus gastroenteritis, prevalence in cases and controls (54 pairs), univariate and multivariate odds ratios using logistic regression and PAR[a]

| | Cases | Controls | | | | | |
|---|---|---|---|---|---|---|---|
| Risk Factor | N (%) | N (%) | OR uni | 95% CI | OR multi | 95% CI | PAR (%) |
| Household member with gastroenteritis | | | | | n.i. | | |
| No | 30 (63.4) | 44 (91.7) | np | | | | |
| Yes, 1 | 10 (21.3) | 4 (8.3) | | | | | |
| Yes, >1 | 7 (14.9) | 0 (0.0) | | | | | |
| Contact with persons with gastroenteritis outside household | | | | | | | 86 |
| No | 13 (24.1) | 33 (61.1) | 1.0 | - | 1.0 | - | |
| Yes | 10 (18.5) | 6 (11.1) | 6.4 | 1.5 to 27.5 | 12.9 | 1.2 to 133.6 | |
| Do not know | 31 (57.4) | 15 (27.8) | 8.2 | 2.3 to 29.0 | 14.8 | 1.8 to 120.6 | |
| Educational level | | | | | n.i. | | |
| Low | 2 (3.7) | 6 (11.1) | 0.3 | 0.0 to 2.9 | | | |
| Middle | 15 (27.8) | 23 (42.6) | 1.0 | - | | | |
| High | 37 (68.5) | 25 (46.3) | 2.1 | 0.9 to 4.6 | | | |
| Food-handling hygiene score[b] | | | 1.2 | 1.0 to 1.6 | 1.5 | 1.1 to 2.1 | 46 |
| Blender in household | 16 (29.6) | 30 (55.6) | 0.2 | 0.1 to 0.7 | 0.1 | 0.0 to 0.6 | |

[a]OR, odds ratio; PAR, population-attributable risk fraction; uni, univariate; multi, multivariate; CI=confidence interval; np, not possible to calculate; n.i.: not in final model.
[b]Higher score indicates less hygienic practices, OR for increase of 1.

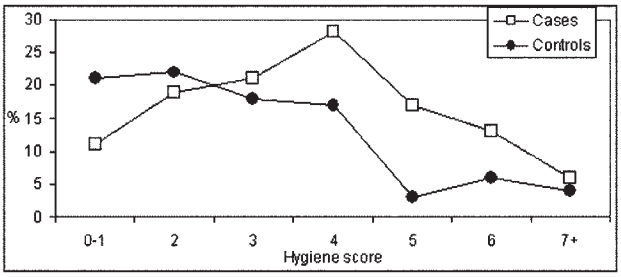


Figure 2. Distribution of food-handling hygiene score in rotavirus gastroenteritis cases (n = 54) and controls (n = 54). (A higher score indicates less hygienic practices.)

of community cases of viral foodborne infection cannot be derived by either approach. However, we strongly support the conclusion that a considerable proportion of NV infections may be prevented by improving food hygiene.

In surveillance systems of outbreaks of NV, person-to-person spread and foodborne spread are reported to be the most common transmission routes (1,8,18). The relative importance of each differs by country and is strongly influenced by the design of the surveillance system (19). Outbreaks covered in a surveillance system do not necessarily represent all outbreaks. Our study shows that in sporadic cases, direct and indirect person-to-person transmission remains the most prominent mode of transmission, followed by food contaminated outside the household. Nevertheless, extrapolation of our estimate to the population of the Netherlands (16 million) suggests that, of the 650,000 NV gastroenteritis cases that occur annually, an estimated 80,000 cases are foodborne, which is more than the estimate for Salmonella (50,000 foodborne cases each year).

No specific food products were associated with NV gastroenteritis. This finding is not remarkable because NV can probably survive on almost all food products that are not cooked before consumption, and a very low infectious dose is required, as has been demonstrated for another naked single-stranded RNA virus, poliovirus (20). Since NV cannot be grown in cell culture, little is known about its heat inactivation profiles. However, studies for another enteric single-stranded RNA virus with similar structure (hepatitis A virus) suggest that heating for 30 s at 90°C will completely inactivate viruses in any food (21). Most published foodborne outbreaks could be traced back to infected food handlers at some point in the production chain, suggesting that this is by far the most common source of foodborne infections (8,22–24). Our results show that, without applying extraordinary hygienic practices but by just following normal hygiene procedures, a substantial portion of sporadic NV infections could be prevented. Because of the high transmission rate in households, persons with a household member with gastroenteritis are at greater risk of being infected. Since several foodborne outbreaks have been reported in which the food handler who had most likely contaminated the food was not symptomatic (yet), making professional food handlers aware of their higher probability of being infected when living with a household member with gastroenteritis might be useful (24).

For NV, for persons of ≥5 years, PAR for food-handling hygiene and the combined PAR for food-handling hygiene and a household gastroenteritis contact were similar. The decrease in OR for a household gastroenteritis contact to almost 1, after hygiene was included in the model, suggests that transmission from one ill household member to another occurs almost entirely through food in persons of ≥5 years. For children, only part of the transmission from one person to another in the household is through food-handling hygiene. Possibly, for young children, exposure is very common and better food-handling hygiene in the household only prevents a minority of exposure possibilities.

A large study in U.S. households showed that the proportion of rotavirus infections acquired in the household was higher for adults than for children, indicating that children introduce the infection into the household (25). In contrast to rotavirus and SLV gastroenteritis, NV gastroenteritis is not limited to the youngest age groups. This finding could explain why food-handling hygiene and having a household gastroenteritis contact had a higher impact on NV gastroenteritis than on SLV gastroenteritis. For rotavirus and SLV, undetected asymptomatic infections (not included as cases in this study) may occur at older age through these routes.

Living in a household with a child attending a daycare center or primary school was univariately associated with NV gastroenteritis. However, when the data were corrected for a household gastroenteritis contact, especially with a child, this association disappeared. This finding suggests that daycare centers and primary schools are the settings in which the primary infection in the household was acquired. The fact that a participant's daycare attendance had only borderline association with NV gastroenteritis indicates that gastroenteritis acquired at daycare centers was less common in our study than secondary transmission in the household.

We could not confirm the association between rotavirus gastroenteritis and daycare center attendance, as has been reported by others (26,27). A study with comparable methods in England also did not find this association (28).

### Methodologic Issues

The optimized score for food-handling hygiene was fitted in the same NV data for which it was used to estimate the effect of hygiene. This might have resulted in an overestimate for NV because noise is also modeled in the pre-

diction. Although all the factors included in the food-handling hygiene index are related to food handling, we cannot exclude the possibility that the index might be a proxy for hygiene as a whole and not just related to food handling. Finally, we assumed that the relationship between the hygiene score and the risk for NV gastroenteritis was exponential. Although this assumption is not entirely true, the exponential model was a good approximation.

In addition, the uncertainty around the estimates will be wide, and PARs should be interpreted as indications of the magnitude of the effect of a risk factor. Many risk factors were tested for, and a type 1 error might have occurred, identifying risk factors that were in fact not associated to the disease. However, for most of the risk factors, plausible biological mechanisms exist. An exception is the association of a blender with rotavirus, on which, therefore, no conclusions are drawn.

By using a case-control design based on clinical gastroenteritis, differentiating risk factors for infection with the virus from risk factors for developing illness after infection is not possible. Factors that represent long-term exposure might have induced immunity earlier in life, and subsequent infections might not result in clinical disease (29,30). As a result, long-term risk factors might be more difficult to detect in case-control studies, or, if the proportion of individual persons with immunity is large, even cause a negative association. Especially for rotavirus, for which immunity is proven to exist, this factor might play a role. Whether relevant immunity to NV and SLV is induced is still under debate.

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Dr. de Wit has worked as an epidemiologist on the epidemiology of gastroenteritis at the National Institute of Public Health and the Environment since 1996.

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Address for correspondence: Yvonne van Duynhoven, Department of Infectious Diseases Epidemiology, National Institute of Public Health and the Environment, P. O. Box 1, 3720 BA Bilthoven, the Netherlands; fax: +31 30 2744409; email: y.van.duynhoven@rivm.nl

# Multidrug-resistant *Mycobacterium tuberculosis* in HIV-Infected Persons, Peru

**Pablo E. Campos,*† Pedro G. Suarez,‡ Jorge Sanchez,†§ David Zavala,‡ Jorge Arevalo,‡ Eduardo Ticona,‡ Charles M. Nolan,*¶ Thomas M. Hooton,* and King K. Holmes***

During 1999 to 2000, we identified HIV-infected persons with new episodes of tuberculosis (TB) at 10 hospitals in Lima, Peru, and a random sample of other Lima residents with TB. Multidrug-resistant (MDR)-TB was documented in 35 (43%) of 81 HIV-positive patients and 38 (3.9%) of 965 patients who were HIV-negative or of unknown HIV status ($p < 0.001$). HIV-positive patients with MDR-TB were concentrated at three hospitals that treat the greatest numbers of HIV-infected persons with TB. Of patients with TB, those with HIV infection differed from those without known HIV infection in having more frequent prior exposure to clinical services and more frequent previous TB therapy or prophylaxis. However, MDR-TB in HIV-infected patients was not associated with previous TB therapy or prophylaxis. MDR-TB is an ongoing problem in HIV-infected persons receiving care in public hospitals in Lima and Callao; they represent sentinel cases for a potentially larger epidemic of nosocomial MDR-TB.

Multidrug-resistant tuberculosis (MDR-TB) threatens global TB control and has been identified in almost all surveyed countries. During 1994 to 1997, the World Health Organization (WHO)-International Union against Tuberculosis and Lung Disease identified high prevalences of MDR-TB in the former Soviet Union, Asia, the Dominican Republic, and Argentina (1).

MDR-TB has been associated with inadequate treatment regimens, poor adherence to treatment, poorly managed TB-control programs, and unenforced hospital infection control programs, as well as HIV infection (2). HIV infection influences the natural history of TB in several ways: active TB occurs within 6 months of acquiring *Mycobacterium tuberculosis* infection in 37% of persons with HIV-induced immunosuppression (3) but occurs in 2% of immunocompetent adults during the first year after acquiring *M. tuberculosis* infection (4). HIV-infected persons with TB also have increased frequency of disseminated and meningeal disease, other extrapulmonary diseases, atypical clinical signs and symptoms, drug-related adverse events, and negative sputum smears for acid-fast bacilli (5).

Nosocomial outbreaks of MDR-TB involving HIV-infected persons have occurred in the United States and other industrialized countries (6–9), but improved treatment and hospital infection control programs have contained these initial outbreaks of MDR-TB. However, such outbreaks are increasingly recognized in countries with more limited resources (10,11).

In Peru, the National TB Control Program received reports of 35,685 new TB cases in 1999 (incidence 141.4/100,000) (12). Passive reporting to the National TB Control Program showed HIV prevalences from 1% to 1.5% in TB patients during 1996 to 1999 (12), but prospective testing of 1,043 patients with a new event of TB in Lima and Callao from September to October 1999 found HIV seropositivity in 2.3% (P.E. Campos, unpub. data). Furthermore, TB was the AIDS-defining illness in 10,939 (28%) AIDS cases in Peru (13), and 50% of persons with AIDS in Peru develop TB at some point in their disease.

A National TB Control Program survey during 1999 found MDR-TB in 57 (3%) of 1,879 Peruvians with a first episode of TB and in 32 (12.3%) of 260 with previously treated TB (14). Of 2,101 TB patients in that survey, 8 had a previous diagnosis of HIV infection. In 1997, at the Dos de Mayo Hospital in Lima, which provides care to the largest number of HIV-infected persons, 9 (34.6%) of 26 HIV-infected patients with TB had MDR-TB (15).

Mortality rates during TB treatment of Peruvian HIV-infected patients has been high; 63%, 51%, 49%, and 39% of patients with a first episode of TB died during treatment

*University of Washington, Seattle, Washington, USA; †Cayetano Heredia University, Lima, Peru; ‡Peruvian Ministry of Health, Lima-Peru; §Asociacion Civil Impacta Salud y Educacion (IMPACTA), Lima, Peru; and ¶Public Health Seattle-King County, Seattle, Washington, USA

that was started in 1996, 1997, 1998, and 1999, respectively (12,16). These rates contrast with a mortality rate of 9% in HIV-infected persons with pulmonary TB in Haiti (17). Treatment regimens prescribed for TB in developing countries are rarely based on susceptibility testing, and since 1987, HIV-infected patients with a first episode of TB in Peru have received a standard course of rifampin, isoniazid, pyrazinamide, and ethambutol in compliance with WHO recommendations (18). Therefore, MDR-TB might contribute to the high mortality rate for TB in HIV-infected Peruvians.

We performed this study to further characterize the prevalence and pattern of MDR-TB in HIV-infected adults in Lima and the adjacent port of Callao in Peru and to explore preventable risk factors that could be used to design, implement, and evaluate better preventive and therapeutic interventions.

## Methods

### Study Design and Population

This study was designed to evaluate the prevalence of MDR-TB in HIV-infected patients ≥18 years of age and to assess potential risk factors for MDR-TB in HIV-infected persons with TB. The study was carried out from February 1999 to January 2000 in Lima and Callao. Lima is the capital city of Peru, and Callao is the adjacent main port; together they have a population of 8,239,891, representing 32% of the national population. Lima and Callao accounted for 78% of all Peruvian AIDS cases reported from 1983 to September 2000 (13) and for 56% of all TB cases reported in 1999 (12). The research protocol for using human participants in this study has been reviewed and approved by the Human Subject Review Committee of the University of Washington and the Ethical Committee of the Scientific Research Office at the Cayetano Heredia University. Informed consent was obtained from the participants.

From February 1999 to January 2000, we introduced an active surveillance system to identify HIV-infected adults with a new event of TB, defined as a first episode of TB or a relapse, at the 10 public hospitals that provide care for most HIV-infected persons living in Lima and Callao. Each of these 10 hospitals has a unit of the National TB Control Program where every patient with a suspected or confirmed diagnosis of TB is referred for further work-up, treatment, follow-up, or referral. To identify HIV-infected patients with a new episode of TB, one trained interviewer periodically visited each of these units. In addition, we encouraged all clinicians to send clinical specimens for isolation of *M. tuberculosis*, and all laboratories to submit such isolates to TB control program reference laboratories for susceptibility testing.

MDR-TB was defined as resistance to both isoniazid and rifampin, with or without resistance to other drugs. Case-patients were adults with previously diagnosed HIV infection and with a new episode of TB, whose isolates of *M. tuberculosis* were MDR-TB. HIV-seropositive controls were adults with previously diagnosed HIV infection and a new event of TB, whose isolates of *M. tuberculosis* were susceptible to isoniazid or rifampin. HIV-positive status was defined by previous, repeatedly positive enzyme-linked immunosorbent assay (ELISA) confirmed by immunofluorescence or Western blot.

From February trough September 1999, a total of 972 adult patients with a new episode of smear-positive TB in Lima and Callao who received care at Ministry of Health facilities were included in the National TB Control Program's surveillance of resistance to anti-TB drugs. Of these 972, a total of 116 had been previously tested for HIV: 7 (6%) were HIV seropositive. From the remaining 965 participants who were HIV seronegative or not tested for HIV infection, we randomly selected a second control group of 153 participants.

### Enrollment, Interview, and Treatment

Both trained interviewers periodically visited each of the 10 hospitals to identify, enroll, and interview HIV-infected patients with a new episode of TB. Because classification as "case" or "control" for HIV-infected patients was determined by drug susceptibility-test results, which became available after 3 or 4 months, each HIV-infected adult with a new episode of TB was eligible to participate, and interviewers were blinded to susceptibility test results. One member of the local TB team introduced the interviewer, who explained the study and invited the patient to participate. Interviewers contacted adults with TB who were HIV seronegative or of unknown HIV status belonging to the HIV–seronegative control group at their homes, where they were invited to participate, enrolled, and interviewed. Patients giving written informed consent underwent a standardized face-to-face interview concerning demographic characteristics, past and current medical history, and potential exposures to *M. tuberculosis* during the 12 months before onset of TB symptoms. Healthcare workers at each facility followed national guidelines of the TB and the AIDS control programs in providing further treatment and follow up of study participants. Data collected were handled exclusively by the study team, ensuring confidentiality.

### Laboratory Methods

Primary isolations were attempted by using Lowenstein-Jensen or Ogawa medium at each hospital laboratory and at local reference laboratories serving the national surveillance study. Susceptibility testing was car-

ried out at the Mycobacteria Laboratory of the Peruvian National Institute of Health, the national reference laboratory for susceptibility testing, or at one of three local mycobacterium reference laboratories. These laboratories used the proportion method to determine the sensitivity profile of each strain (19), using the following critical concentrations (μg/mL): isoniazid, 0.2; rifampin, 40; streptomycin, 4; ethambutol, 2; and pyrazinamide, 100. The National Reference Laboratory underwent external quality control by the Pan American Health Organization/WHO Instituto Panamericano de Protección de Alimentos y Zoonosis (INPPAZ) and performed quality control for the three local reference laboratories (20).

### Data Analyses

SPSS 10.0 software (SPSS, Inc., Chicago, IL) was used for data entry and analyses. Percentages of MDR-TB were compared in HIV-positive and HIV-negative patients with TB, and risk factors for MDR-TB in HIV-infected patients were explored by calculation of odds ratios (OR) for dichotomous variables. Medians of continuous variables were compared by using Student t test for independent samples.

## Results

### Characteristics of Patients with TB

Of 415 HIV-seropositive patients diagnosed and reported with a new episode of TB (on the basis of acid-fast smear or culture results or on clinical characteristics) at the 10 hospitals during the study period, 157 (38%) were not interviewed: 87 had already left the hospital and could not be located at referral health centers, 67 were in poor clinical condition and unable to answer the questionnaire, and 3 declined to participate. The remaining 258 seropositive participants interviewed averaged 32 years of age (range 18 to 62) and reported 9.8 years of education; 77% were men, 57 % single, and 20% had had at least one previous episode of TB. Isolation of *M. tuberculosis* was attempted in 239 (93%); 135 were culture-positive, 61 were smear positive for acid-fast bacilli, and 62 were diagnosed on the basis of clinical criteria. Drug-susceptibility testing was requested for all positive cultures and completed on 81, who were the focus of subsequent analyses; the remaining strains were lost before or during transport to the National Reference Laboratory for susceptibility testing.

Of the 153 TB patients randomly selected as controls without known HIV infection, we located 110; 108 agreed to participate. They averaged 29.3 years of age (range 18 to 80) and reported 10.3 years of education; 57% were men, 52% single, and 9.3% had had at least one previous episode of TB.

Table 1 presents characteristics of participants with and without known HIV infection for whom drug-susceptibility testing was completed. Because HIV infection in Peru disproportionately affects men who have sex with men, HIV-seropositive persons were more often men and less often married, divorced, or widowed; family income and family ownership of the home were more common among those not known to have HIV infection. More importantly, previous history of TB or TB prophylaxis was significantly more common in HIV-infected participants, as was history of contact with hospitals.

### Drug-Susceptibility Test Results

The prevalence of MDR-TB was 43% in the 81 HIV-seropositive patients with available susceptibility test results and 3.9% in the 965 patients whose HIV status was negative or unknown ($p < 0.001$); only 1 (0.9%) of 108 patients whom we randomly selected and interviewed from this group of 965 TB patients had an MDR-TB isolate (Table 2). The prevalence of resistance to any drug was also higher in *M. tuberculosis* isolates from HIV-positive patients than in isolates from the 965 without known HIV infection.

Drug-susceptibility test results were available for 26 (16.6%) of the 157 HIV-seropositive patients we did not interview; 35% of these patients had MDR-TB, not significantly different from the 43% of those we did interview ($p = 0.44$).

### HIV Patients with MDR-TB and with Non–MDR-TB

Of HIV-seropositive participants with TB (Table 3), MDR-TB was significantly associated with TB diagnosed at hospital A (OR 3.7, 95% confidence interval [CI] 1.3 to 10), with employment during the 12 months before the onset of symptoms, and with exposure to TB at work, but not with age, sex, marital status, education level, crowding in the home, or low income. MDR-TB was not significantly associated with previous episodes of TB or with TB prophylaxis; the proportion who had received TB prophylaxis or had a previous episode of treatment for TB was 17 (49%) of 35 with MDR-TB versus 20 (43%) of 46 without MDR-TB (OR 1.2, 95% CI 0.5 to 3.0).

### Hospital Characteristics

Table 4 lists the 10 major public hospitals in Lima and Callao by number of persons with newly diagnosed AIDS reported during 1999, number with TB diagnosed during 1999, number of HIV-infected patients with a new event of TB during the study, and percentage of isolates of *M. tuberculosis* identified as MDR-TB. All but one of the MDR-TB cases were identified in the three hospitals reporting the largest number of HIV-seropositive patients with a new event of TB during 1999.

Table 1. Demographic characteristics, past medical history, and potential exposures to tuberculosis (TB) of participants with and without known HIV infection[a]

| Variable | HIV-infected (n = 81) Mean ± SD, or % | Negative or unknown HIV status (n = 108) Mean ± SD, or % | p value or OR (95% CI)[b] |
|---|---|---|---|
| Age | 31.6 ± 7.0 | 29.3 ± 11.9 | 0.13 |
| Male | 80 | 57 | 3.0 (1.6 to 5.9) |
| Marital status | | | |
| Single | 59 | 52 | 0.42 |
| Married | 9 | 20 | 0.05 |
| Divorced/widowed | 11 | 28 | <0.01 |
| Cohabitant | 21 | 0 | <0.01 |
| Y of education | 9.6 ± 2.8 | 10.3 ± 2.1 | 0.08 |
| No. residents/no. bedrooms | 3.0 ± 2.0 | 2.8 ± 1.4 | 0.48 |
| Family income | 603 ± 333 | 845 ± 404 | <0.01 |
| Family owns home | 75 | 88 | 0.4 (0.2 to 0.9) |
| Employed[d] | 63 | 28 | 4.4 (2.4 to 8.2) |
| Exposure to TB at work[c] | 17 | 3 | 7.3 (2.0 to 26.4) |
| Exposure to TB at home[c] | 16 | 1 | 20 (2.6 to 160) |
| Previously treated TB[e] | 27 | 9 | 3.6 (1.6 to 8.3) |
| Ambulatory care at health centers[d] | 21 | 5 | 5.5 (1.9 to 16) |
| Ambulatory care at hospitals[d] | 40 | 1 | 70 (9.3 to 526) |
| Inpatient care[d] | 14 | 0 | <0.001 |
| Inpatient MoH hospitals[d] | 11 | 0 | <0.001 |
| Any exposure to hospital[d] | 44 | 1 | 86 (11.4 to 644) |
| TB prophylaxis[e] | 22 | 0 | <0.001 |

[a]OR, odds ratio; CI, confidence interval; MoH, Ministry of Health.
[b]p values proved when comparing means or when one column had a value of 0.
[c]Exposure to someone with TB at work or at home during the 12 months before onset of symptoms.
[d]Variables reported by patients to have occurred during the 12 months before onset of symptoms.
[e]Ever in the past.

### Assessment of Possible Bias

Interviewed patients with (N = 81) or without (N = 177) susceptibility results were similar with respect to age, years of education, family income, symptom duration, time since previous TB, TB prophylaxis in the past, crowding in the home, gender, marital status, home ownership, and employment; and during the past 12 months, exposure to TB at work, exposure at home, ambulatory care at health centers, ambulatory care at hospitals, inpatient hospital care, and participation in HIV or TB support groups. History of previous TB was somewhat more frequent in those tested (27%) than in those not tested (17%, p = 0.09), and history of *Pneumocystis carinii* pneumonia (PCP) prophylaxis was more frequent among those tested (50%) than in those not tested (32%, p < 0.01).

## Discussion

This study documents high rates of MDR-TB in HIV-infected persons with TB receiving care at public hospitals in Lima and Callao; MDR-TB was 11 times more common in these patients than in 965 TB patients without known HIV infection in Lima and Callao. In comparison to persons having TB without known HIV infection, the HIV-infected patients with TB had higher frequency of contact with health centers and hospitals, and MDR-TB was found mainly in patients receiving care at the three hospitals serving the largest number of HIV-infected patients with TB. Although participants with HIV infection more often had had previous episodes of TB, and presumably, only the HIV-infected persons had received TB prophylaxis, these factors were not significantly associated with MDR-TB in the HIV-infected patients with TB.

The 43% prevalence of MDR-TB among HIV-infected persons with TB found in this study is higher than the 36% previously found in Italy (21), and the 28.3% found in Argentina, both during epidemics of nosocomial MDR-TB (1).

Because early clinical manifestations of TB often develop in HIV-infected persons (3,5), they mark sentinel cases that first indicate outbreaks of nosocomial transmission of TB and may represent a larger number of nosocomial transmission of TB.

Three factors drive TB transmission: the rate of exposure of susceptible to infectious persons, the efficiency of transmission per exposure, and the average duration of infectiousness once infection has occurred (22). For HIV-infected persons, higher exposure to TB in general could easily result from routine periodic visits to clinical settings also frequented by patients with TB (as occurs in Lima and Callao, where most persons with HIV infection attend HIV/AIDS clinics located at large hospitals); or from the mixing of HIV-infected patients with TB patients in these settings because of the archaic practice of mixing patients who have communicable diseases (like TB and HIV infec-

Table 2. Drug-susceptibility profiles of *Mycobacterium tuberculosis* isolates from participants with and without known HIV infection

| | | HIV negative or unknown status | |
|---|---|---|---|
| | HIV positive patients | Interviewed patients | All patients |
| Characteristic | N (%) | N (%) | N (%) |
| Total no. tested for drug resistance | 81 (100) | 108 (100) | 965 (100) |
| Fully susceptible isolates | 28 (35) | 92 (85) | 789 (82) |
| Any resistance | 52 (65) | 16 (15) | 76 (18) |
| Any H resistance | 42 (52) | 11 (10) | 110 (10) |
| Any R resistance | 39 (48) | 2 (2) | 53 (6) |
| Any E resistance | 19 (24) | 0 (0) | 27 (3) |
| Any S resistance | 39 (48) | 12 (11) | 88 (9) |
| Any Z resistance | 28 (35) | NA | NA |
| Multidrug resistance | 35 (43) | 1 (1) | 38 (3.9) |
| Only HR resistance | 3 (4) | NA | NA |
| HRZ resistance | 25 (32) | NA | NA |
| HRESZ resistance | 11 (14) | NA | NA |

[a]NA, not available; H, isoniazid; R, rifampin; E, ethambutol; S, streptomycin; Z, pyrazinamide; HRZ resistance includes any strain resistant at least to H and R and Z.

tion) in large multiple-bed wards. MDR-TB case-patients tend to be selectively hospitalized because they do not respond to routine therapy.

Conditions that increase exposure to TB, such as overcrowding, long waiting times in clinics, sharing of facilities, and large open multiple-bed wards, are common in medical institutions in the developing world. Increased efficiency of transmission per exposure could occur if HIV-related immunosuppression increases host susceptibility to acquisition of infection by *M. tuberculosis* in general or by MDR-TB strains of TB in particular.

Several factors could prolong the duration of infectiousness of MDR-TB in HIV-immunosuppressed persons, including continued use of isoniazid- and rifampin-based regimens as initial therapy for TB in HIV-infected persons, even during ongoing outbreaks of MDR-TB transmission. Patients receiving ineffective treatment will not improve and will more often use clinical services as outpatients and as inpatients, increasing the exposure to MDR-TB of other patients. In addition, increased frequency of atypical clinical pictures and smear-negative results, relatively common features in HIV-infected patients with TB, can contribute to delayed diagnosis and prolonged infectivity.

Evidence suggests that HIV infection favors the emergence of acquired drug resistance in individual patients during treatment (23,24). HIV-infected persons have a higher risk for acquisition of isolated rifampin resistance (25,26), and once- or twice-weekly rifamycin-based regi-

Table 3. Demographic characteristics, past medical history, and potential exposures to TB of HIV-infected participants with MDR-TB and without MDR-TB[a]

| | HIV-infected with MDR-TB (n = 35); | HIV-infected without MDR-TB (n = 46); | | |
|---|---|---|---|---|
| Variable | Mean ± SD or % | Mean ± SD or % | p value | OR (95% CI) |
| Employed[b] | 46 | 76 | | 0.3 (0.1 to 0.7) |
| Exposure to TB at work[c] | 6 | 26 | | 0.2 (0.04 to 0.8) |
| Exposure to TB at home[c] | 20 | 13 | | 1.7 (0.5 to 5.5) |
| Previously treated TB[d] | 29 | 26 | | 1.1 (0.4 to 3.0) |
| Duration of symptoms | 6.7 ± 5.4 | 7.8 ± 6.6 | 0.44 | |
| Ambulatory care at health centers[b] | 14 | 26 | | 0.5 (0.2 to 1.5) |
| Ambulatory care at hospitals[b] | 43 | 37 | | 1.3 (0.5 to 3.1) |
| Ambulatory care at hospitals[e] | 3924 ± 5749 | 2737 ± 3361 | 0.48 | |
| Inpatient care[b] | 17 | 11 | | 1.7 (0.5 to 6.1) |
| Inpatient MoH hospitals[b] | 11 | 11 | | 1.1 (0.3 to 4.3) |
| Days of hospitalization[b] | 18 ± 11 | 30 ± 26 | 0.36 | |
| Diagnosed at hospital "A" | 80 | 52 | | 3.7 (1.3 to 10) |
| Any exposure to hospital[b] | 51 | 39 | | 1.6 (0.7 to 4.0) |
| HIV support group[b] | 11 | 7 | | 1.9 (0.4 to 8.9) |
| TB prophylaxis[d] | 26 | 20 | | 1.4 (0.5 to 4.1) |
| Mo. of TB prophylaxis | 6.7 ± 5 | 4.4 ± 3.3 | 0.27 | |
| PCP prophylaxis[d] | 57 | 44 | | 1.7 (0.7 to 4.2) |
| Extrapulmonary TB | 29 | 44 | | 0.5 (0.2 to 1.3) |

[a]MDR-TB, multidrug-resistant tuberculosis; OR, odds ratio; CI, confidence interval; MoH, Ministry of Health; PCP, *Pneumocystis carinii* pneumonia.
[b]Variables reported by patients to have occurred 12 months before onset of symptoms.
[c]Exposure to someone with TB at work or at home 12 months before onset of symptoms.
[d]Ever in the past.
[e]As a continuous variable: total minutes of exposure 12 months before onset of symptoms.

Table 4. Ranking of hospitals by number of newly diagnosed cases of AIDS, tuberculosis (TB), HIV, and TB co-infection, February 1999–January 2000[a,b]

| Hospital | No. AIDS patients reported during 1999[c] | No. TB patients diagnosed during 1999 | Total no. HIV-infected patients with a new diagnosis of TB | No. (%) HIV-infected patients with a new diagnosis of TB interviewed | No. MDR-TB ÷ total drug susceptibility test results available (%) |
|---|---|---|---|---|---|
| A | 154 | 1,985 | 204 | 116 (57) | 28/52 (54) |
| B | 60 | 723 | 49 | 40 (82) | 4/9 (44) |
| C | 139 | 1,068 | 41 | 24 (59) | 2/7 (29) |
| D | 10 | 891 | 37 | 29 (78) | 0/4 (0) |
| E | 33 | 635 | 33 | 21 (64) | 0/5 (0) |
| F | 95 | 608 | 29 | 11 (38) | 0/1 (0) |
| G | 9 | 425 | 12 | 11 (92) | 0/1 (0) |
| H | 30 | 169 | 6 | 4 (67) | 0/1 (0) |
| I | 4 | 130 | 3 | 1 (33) | 0/0 (NA) |
| J | 2 | 131 | 1 | 1 (100) | 1/1 (100) |
| Total | 554 | 6,765 | 415 | 258 | 35/81 (43) |

[a]Among those co-infected with TB and HIV, the number (%) with MDR-TB.
[b]MDR-TB; multidrug-resistant tuberculosis.
[c]Number of AIDS cases reported to the National AIDS and STD Control Program.

mens increase the risk for acquired rifamycin resistance in TB patients with advanced HIV disease (27–29). MDR-TB is more often a consequence of addition of rifampin resistance than of addition of isoniazid resistance (22).

Limitations of this study included the fact that TB cases with HIV infection were recruited from hospitals where patients with known HIV infection receive care in Lima, whereas HIV-negative controls were recruited from non-hospital clinics. However, methodologic features and results of this evaluation suggest that results may be generalizable to all HIV-infected patients with TB receiving care within the Ministry of Health system in Lima and Callao. Of 457 new episodes of TB in HIV-infected persons ≥18 years in Lima and Callao during 1999 (12), we identified 415 cases (91%) through the TB units at each of these 10 hospitals during the 12-month study. We systematically interviewed HIV-infected patients well in advance of knowing whether they had MDR-TB, and we systematically interviewed a random sample of TB patients without known HIV infection from a larger representative sample of TB patients from Lima and Callao. Participants with known HIV infection were selected consecutively and patients without known HIV infection were selected randomly from all case-patients of TB receiving care at public health services. Comparisons of those interviewed versus not interviewed, and those tested and not tested for drug susceptibility suggested no bias, except that those tested had somewhat higher frequencies of previous TB and of PCP prophylaxis. The latter could reflect greater immunosuppression or greater contact with the medical care system. Although both could have biased results toward higher estimates of MDR-TB prevalence among HIV-seropositive patients, a previous history of TB or of TB prophylaxis was not associated with MDR-TB.

We were unable to interview all HIV-positive patients with TB diagnosed during the study, raising the question of whether the prevalence of MDR-TB was higher in those we interviewed than in those not interviewed. However, the 43% frequency of MDR-TB in those we interviewed did not differ significantly from the 35% prevalence of MDR-TB in the 26 patients who we did not interview for whom susceptibility testing was performed. Finally, concerning the fact that we did not obtain HIV serologic finding from all of the 108 controls with negative or unknown HIV status, if the prevalence of HIV had been higher than the 2.3% prevalence observed during prospective testing in Lima in 1999, such misclassification bias would only have reduced the differences observed between groups.

Community-based studies throughout the United States have documented increased prevalence of MDR-TB in HIV-infected people (2), and in a recent report of several surveys (30), MDR-TB was found more often in HIV-infected patients than in HIV-uninfected patients, but after adjustment for previous treatment for TB the difference between HIV-infected and HIV-uninfected patients was no longer statistically significance. However, in our survey, even after eliminating patients with history of prior episodes of TB or TB prophylaxis, MDR-TB was still seen in 18 (43%) of 44 isolates from HIV-infected persons versus 24 (3%) of 814 HIV-negative controls ($p < 0.001$).

## Conclusion

Although rigorous compliance with infection control recommendations (31), particularly those related to engineering control, is difficult in developing countries, the combined epidemics of TB, MDR-TB, and HIV/AIDS make infection control measures essential. WHO guidelines recommend a hierarchy of controls (administrative, environmental, and personal respiratory protection), many of which entail little or no cost (32). Minimal measures must include strict respiratory isolation for patients with confirmed or suspected TB and mandatory wearing of appropriate masks for persons entering all patient rooms, for patients leaving their rooms when unavoidable, and for patients with cough when seen in clinics. The efficacy of

these essential measures to avert TB transmission in healthcare settings have been demonstrated (33). Segregating persons with TB from those with HIV in individual rooms with negative air flow, establishing safer sputum sampling collection procedures, improving the laboratory support for early identification of TB and of MDR-TB, and providing more effective treatment regimens to patients at increased risk for MDR-TB are necessary. HIV testing of patients with TB and susceptibility testing of *M. tuberculosis* isolates from HIV-infected patients should be routine in settings where outbreaks or endemic transmission of MDR-TB is occurring in HIV-infected patients.

Nosocomial MDR-TB transmission at hospital A has been ongoing since 1997. Recently published IS6110 restriction fragment length polymorphism analysis of *M. tuberculosis* strains collected between July 1997 and April 1999 and belonging to HIV-positive inpatients clearly implicate nosocomial transmission of MDR-TB in this hospital (34). Similarly, outbreaks of nosocomial MDR-TB in HIV-infected persons have emerged first in New York, Buenos Aires, and Lima (settings providing hospital-based care, including antiretroviral therapy, for HIV-infected persons). Unrecognized MDR-TB outbreaks in other developing countries are likely. As delivery of antiretroviral therapy for HIV in developing countries proceeds, nosocomial exposure of HIV-infected persons to TB must be minimized. More effective surveillance, prevention, and treatment for MDR-TB are essential.

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Dr. Campos is an associate professor at the *Cayetano Heredia* School of Public Health. He has worked in HIV prevention and care since 1990.

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Address for correspondence: King K. Holmes, Center for AIDS and STD, University of Washington, Box-359931, 325 9th Ave. Seattle, WA 98104, USA; fax: 206-731-3694; email: worthy@u.washington.edu

# Human Monocytotropic Ehrlichiosis, Missouri

**Juan P. Olano,* Edwin Masters,† Wayne Hogrefe,‡ and David H. Walker***

To determine the incidence, clinical and laboratory characteristics, and utility of molecular diagnosis of human monocytotropic ehrlichiosis (HME) in the primary care setting, we conducted a prospective study in an outpatient primary care clinic in Cape Girardeau, Missouri. One hundred and two patients with a history of fever for 3 days (>37.7°C), tick bite or exposure, and no other infectious disease diagnosis were enrolled between March 1997 and December 1999. HME was diagnosed in 29 patients by indirect immunofluorescent antibody assay and polymerase chain reaction (PCR). Clinical and laboratory manifestations included fever (100%), headache (72%), myalgia or arthralgia (69%), chills (45%), weakness (38%), nausea (38%), leukopenia (60%), thrombocytopenia (56%), and elevated aspartate aminotransferase level (52%). Hospitalization occurred in 41% of case-patients. PCR sensitivity was 56%; specificity, 100%. HME is a prevalent, potentially severe disease in southeastern Missouri that often requires hospitalization. Because clinical presentation of HME is nonspecific, PCR is useful in the diagnosis of acute HME.

Ehrlichioses were recognized as causing human infectious diseases relatively recently. Ehrlichiae, obligately-intracellular gram-negative bacteria, have evolved in close association with a vector arthropod and a zoonotic host and have been traditionally recognized as veterinary pathogens (1–4). In the United States, the first human case of ehrlichiosis was reported in 1987 (4). In 1991, the agent was isolated and recognized as a novel pathogen, *Ehrlichia chaffeensis* (5). By 1997, 742 cases in 47 states had been reported to the Centers for Disease Control and Prevention, most likely an underestimate of the true incidence (6). Passive reporting of cases has yielded the concept that even in the states with the most cases the incidence is low (e.g., 0.5 cases/100,000 persons in Arkansas). The clinical spectrum of human monocytotropic ehrlichiosis (HME) ranges from mild to a life-threatening multisystem disease (7–11) with a case-fatality rate of 2% to 3% and a duration of illness in the absence of antiehrlichial treatment averaging 3 weeks. The clinical manifestations are neither sensitive nor specific for the diagnosis of HME. Sequelae include asthenia that can continue months after recovery and an ill-defined immunosuppression that predisposes the patient to opportunistic infections. Conversely, *E. chaffeensis* can cause overwhelming infection in patients with AIDS or other immunosuppressive conditions (12–14).

The exploding population of the natural reservoir of *E. chaffeensis,* white-tailed deer, and the expansion of the range and population of the vector tick *Amblyomma americanum* are important ecologic factors in the continuing emergence of HME (15–19). Other tick-borne human granulocytotropic infections are caused by *Anaplasma phagocytophilum* and *E. ewingii*.

Although HME was described more than a decade ago, prospective studies are scarce (8,20–22). The present investigation describes the first office-based, prospective study of HME in the primary care setting, an investigation over a period of 3 years in southeast Missouri.

## Materials and Methods

### Epidemiologic and Clinical Data

The study area included Cape Girardeau and surrounding counties in southeast Missouri and southwestern Illinois. Approximately 100,000 persons were covered by the health services offered by the medical community. Patients were enrolled from March 1997 through December 1999. The clinical definition of a potential HME case-patient was a patient who had had fever (≥37.7°C) for ≥3 days, possible tick bite or other tick exposure, and no other infectious disease diagnosis established. The patients were given two questionnaires, one during the acute phase of the disease and the second during the convalescent phase when the diagnosis of HME was confirmed by appropriate laboratory studies. A third questionnaire was given to the primary care provider. The information requested included the following: age, gender, occupation, tick exposure/bites, clinical signs and symp-

*University of Texas Medical Branch, Galveston, Texas, USA; †Premier Family Physicians, Cape Girardeau, Missouri, USA; and ‡Focus Technologies, Cypress, California, USA

toms, duration of symptoms, occurrence and duration of hospitalization, antibiotic treatment, days of treatment until resolution of fever, and laboratory data. The protocol study was approved by the Institutional Review Board of the University of Texas Medical Branch.

### Statistical Analysis

All patient information and laboratory results were entered into Microsoft Excel worksheets (Microsoft Corp., Redmond, WA). Data were analyzed by using Sigma Stat Version 2.03 (SPSS Inc., Chicago, IL).

### Laboratory Case Definition Criteria

#### Definite and Probable HME Cases

A definite HME case was defined as follows: Patients who met the clinical definition and had one of the following conditions: a) serologic immunoglobulin (Ig) G rise from <1:64 to ≥1:64 with a positive polymerase chain reaction (PCR) result, or b) IgG seroconversion (fourfold rise) to ≥1:128 without positive PCR or c) positive PCR results in two separate laboratories or for at least two target genes, or d) single serum Ig G titer of ≥1:256, or e) positive culture for *E. chaffeensis*.

A probable case of HME was defined as follows: Patients who met the clinical definition and had a) single IgG titers of 1:64 or 1:128, or b) positive PCR results in one laboratory for only one target gene.

### Processing of Blood Samples

The samples were collected in EDTA-containing tubes and shipped in wet ice overnight to the University of Texas Medical Branch in Galveston. The blood elements were separated by differential gradient centrifugation with Ficoll-Hypaque. The mononuclear band was harvested, washed twice in phosphate-buffered saline (PBS), and resuspended in 2 mL of PBS; 500 µL was then added to DH82, THP-1, and HL-60 cell cultures. The remaining 500 µL was saved for PCR analysis. Serum samples were received separately in red-topped tubes and kept at –20°C until antibody analysis was performed.

### Indirect Immunofluorescent Antibody Assays (IFA)

Serum specimens were screened at 1:64 dilution, according to a previously published protocol (23). Positive serum specimens were diluted serially in twofold increments to 1:4,096. The highest dilution with a 1+ intensity of fluorescent staining was considered the end-point titer. HL60 cells infected with *A. phagocytophilum* (Webster strain) were also used for IFA testing for human granulocytic anaplasmosis (HGA). The cut-off values for HGA testing were set at 1:80, and the samples were serially diluted to 1:1280.

### Preparation of DNA

DNA was extracted from the harvested mononuclear band by using the IsoQuick Extraction kit (ORCA Research, Bothell, WA) during the first year of the study and with the QIAgen DNA extraction kit (QIAgen, Santa Clarita, CA), according to the manufacturer's instructions, during the remaining 2 years.

### PCR Reactions

#### 16S rRNA Subunit Gene

For the first-stage amplification of this gene, a 100-µL reaction mixture containing 10 µL of DNA template, 75 µL of sterile $H_2O$, 10 µL of 10X PCR buffer (Boehringer Manheim, Indianapolis, IN), 1 µL of primers ECB and ECC (Table 1) at a final concentration of 1 µM each, 2 µL of deoxynucleotide triphosphates (final concentration, 200 µM), and 1 µL of *Taq* polymerase (Boehringer Manheim, Indianapolis, IN; final concentration 2.5 U). For nested PCR, 1 µL of each first-stage amplification reaction was amplified in a second 100-µL reaction tube after careful manipulation of the specimens in an AirClean 600 Workstation (AirClean Systems, Raleigh, NC) and aspiration of the PCR mixture with cotton-filled tips. The conditions were the same except for the use of species-specific primers for *E. chaffeensis*, HE1 and HE3 (Table 1).

#### 120-kDa Protein Gene

The first-stage amplification reactions contained the same reagents as described above with the exception of *E. chaffeensis* species-specific primers for the 120-kDa protein gene, PXCF3 and PXAR4. One microliter was then amplified with nested primers for the 120-kDa protein gene with primers PXCF3b and PXAR5 (Table 1).

#### *nad* A Gene

The first-stage amplification was done under the same conditions as described for the other genes with primers ECHNADA1 and PXCR6. One microliter was then amplified in a second 100-µL-reaction tube with nested primers specific for the *nad A* gene of *E. chaffeensis* NADPCR and PXCR7 (Table 1).

#### 16S rRNA gene for HGA

The first-stage amplification reactions contained the same reagents as described above with the exception of the universal eubacterial primers for the 16S rRNA subunit gene, PC5 and Pomod. One microliter was then amplified with nested primers specific for *A. phagocytophilum*, GE9f, and GE10r (Table 1).

All reactions were performed in a PowerBlock II System (Ericomp Inc., San Diego, CA). The PCR products

Table 1. List of PCR primers used in this study for amplification of ehrlichial DNA sequences from blood specimens, Cape Girardeau, Missouri, 1997–1999

| Target gene | Outside primer pair | Nested primer pair | Cycles: T° (time)[a] for outside primers | Cycles: T° (time)[a] for nested primers |
|---|---|---|---|---|
| 16S rRNA subunit gene *Ehrlichia chaffeensis* | ECB 5′CGTATTACCGCGGCTGCTGGA-3′ | HE1 5′′CAATTGCTTATAACCTTTTCCTTATAAAT-3′ | 94 (60) 45 (120) 72(60) | 94 (60) 55 (120) 72(60) |
| | ECC 5′AGAACGAACGCTGGCGGCAGCC-3′ | HE3 5′TATAGGTACCGTCATTATCTTCCCTAT-3' | | |
| 120-kDa protein gene *E. chaffeensis* | PXCF3 5′GAGAATTGATTGTGGAGTTGG-3′ | PXCF3b 5′-CAGCAAGAGCAAGAAGATGAC-3′ | 94(60) 48(120) 72(60) | 94(60) 54(120) 72(60) |
| | PXAR4 5′ACATAACATTCCACTTTCAAA-3′ | PXAR5 5′ATCT′ | | |
| *nadA* gene *E. chaffeensis* | ECHNADA1 5′-TCATTTCGTGCTTTCTTATTG-3′ | NADPCR 5′ACGTCATTTGGCTCAGGA-3′ | 94(60) 48(120) 72(60) | 94(60) 48(120) 72(60) |
| | PXCR6 5′-CAAACGCATATG TGGGCA-3′ | PXCR7 5′-TGTCGATCCAATGAAAT GAGC-3′ | | |
| 16S rRNA subunit gene. *Anaplasma phagocytophilum* | PC5 5′-TACCTTGTTACGACTT-3′ | GE9f 5′-AACGGATTATTCTTTATAGCTTGCT-3′ | 94(60) 38(120) 72(60) | 94(60) 60(120) 72(60) |
| | Pomod 5′-AGAGTTTGATCCTGG-3′ | GE10r 5′-GGAGATTAGATCCTCTTAACGGAA-3′ | | |

[a] Temperature sequence: Denaturing, annealing and synthesis. Time given in seconds. All polymerase chain reactions (PCR) were performed for 35 cycles.

were then separated electrophoretically at 100V for 30 to 40 min in a 1.5% agarose gel and then stained with ethidium bromide. The gel was then examined under ultraviolet light.

### Sequence Analysis

The PCR products were purified by QIAquick (QIAgen, Santa Clarita, CA). The nucleotide sequence was then determined by the dideoxynucleotide method of cycle sequencing with *Taq* polymerase (ABI Prism 377 DNA sequencer, Perkin-Elmer Corp., Foster City, CA). The sequencing reaction was carried out for each strand of DNA to avoid possible errors of incorporation of nucleotides by *Taq* polymerase. The sequences were analyzed by Genetics Computer Group, Wisconsin Package software and by Lasergene software (DNA Star, Inc., Madison, WI).

### Cultivation

Ehrlichial isolation was attempted by adding DH82, THP-1, and HL-60 cell lines as described above. The flasks were fed every 3–4 days as needed and kept for up to 60 days at 37°C and 5% $CO_2$. Samples of the cell monolayers or suspensions were stained with DiffQuik weekly and evaluated for the presence of intracellular morulae. At the end of 60 days, and before discarding the flasks, DNA was extracted from the cell monolayers or flasks as described above. PCR was then performed with 16S rRNA ehrlichial primers that were used for the first-stage reactions described above.

## Results

### Demographic Findings

A total of 102 patients met the clinical definition criteria and were enrolled in the study during the 3-year period (three full tick seasons). HME was diagnosed in 29 patients on the basis of the defined criteria (case-patients, Table 2). Twenty-five of these cases were considered definite, and four were considered probable. Six cases were diagnosed in 1997, 14 in 1998, and nine in 1999. Seronegative patients from whom convalescent-phase serum samples were not obtained were excluded from the study as well as those who did not answer the questionnaires (53 patients). Of the 49 case-patients that were included in the final analysis, paired-serum samples were available in 33 cases. Twenty of these case-patients did not show seroconversion and therefore comprised the control group (noncase-patients). Twenty-one case-patients (72%) were male and eight case-patients (28%) were female. The age of the patients ranged from 15 to 78 years (mean: 48.2 years). The mean age for men was 48.8 years and for women, 46.1 years. Ages ranged from 15 to 70 years for men and 22 to 78 years for women. Twenty-three case-patients (79%) lived in a southeast Missouri county (Cape Girardeau, Bollinger, Scott, Stoddard, Phelps, and Perry) and six case-patients (21%) lived in a southwestern Illinois county (Union, Jackson and Johnson) (Figure). A tick bite was documented in 21 case-patients (72%), and tick exposure without a tick bite in 8 case-patients (28%). For all case-patients, tick attachment ranged from 24 to 72 hours, except for one case-patient who experienced tick attachment for 12 hours. The incubation period from observed tick bite until onset of illness ranged from 1 to 4 weeks. All cases occurred between April and mid-August. Two cases (7%) occurred in the month of August, five cases (17%) in May, three cases (10%) in both June and April, and 16 (56%) in July.

### Clinical and Laboratory Findings

A total of 29 cases were diagnosed with HME by IFA, PCR, or both. The clinical signs and symptoms associated

Table 2. Selected epidemiologic and laboratory results for 29 patients with human monocytotropic ehrlichiosis (HME). Cape Girardeau, 1997–1999

| Patient no. | Age (y) | Sex | Y of diagnosis | PCR result[a] | IFA titer acute phase | IFA titer convalescent | WBC x $10^9$/L | Platelets x $10^9$/L |
|---|---|---|---|---|---|---|---|---|
| 1 | 44 | M | 1999 | + | 1:512 | 1:1024 | 1.9 | 90 |
| 2 | 42 | M | 1999 | + | 1:256 | 1:512 | 3.5 | 114 |
| 3 | 63 | F | 1999 | – | 1:1024 | 1:2048 | 6.4 | 83 |
| 4 | 53 | M | 1999 | + | Neg | 1:512 | 4.5 | 180 |
| 5 | 77 | F | 1999 | – | 1:1024 | NA | 3.5 | 44 |
| 6 | 43 | M | 1999 | + | 1:512 | NA | 1.9 | 89 |
| 7 | 48 | M | 1999 | + | 1:128 | 1:2048 | 4.0 | NA |
| 8 | 30 | M | 1999 | – | 1:1024 | NA | NA | NA |
| 9 | 28 | F | 1999 | + | 1:512 | NA | 5.4 | NA |
| 10 | 22 | F | 1998 | + | Neg | 1:128 | 2.1 | 142 |
| 11 | 59 | M | 1998 | + | Neg | 1:256 | 8.8 | 229 |
| 12 | 67 | M | 1998 | + | Neg | 1:512 | 4.2 | 36 |
| 13 | 78 | F | 1998 | + | Neg | NA | NA | NA |
| 14 | 49 | F | 1998 | + | 1:4096 | 1:4096 | 2.6 | 271 |
| 15 | 65 | M | 1998 | – | 1:256 | 1:1024 | 4.3 | 207 |
| 16 | 26 | M | 1998 | – | 1:1024 | NA | 2.9 | 106 |
| 17 | 44 | F | 1998 | – | 1:64 | NA | 10.0 | 397 |
| 18 | 27 | M | 1998 | – | 1:64 | NA | 8.5 | 246 |
| 19 | 24 | F | 1998 | + | 1:256 | NA | 2.4 | 69 |
| 20 | 59 | M | 1998 | – | 1:1024 | NA | 4.9 | 102 |
| 21 | 65 | M | 1998 | – | 1:256 | NA | 4.4 | 121 |
| 22 | 52 | M | 1998 | – | 1:1024 | 1:1024 | 1.2 | 39 |
| 23 | 54 | M | 1998 | + | 1:128 | NA | NA | NA |
| 24 | 15 | M | 1997 | – | Neg | 1:64 | 6.4 | 308 |
| 25 | 70 | M | 1997 | + | Neg | NA | 5.0 | 222 |
| 26 | 47 | F | 1997 | + | Neg | 1:512 | NA | NA |
| 27 | 31 | M | 1997 | + | Neg | NA | 3.5 | 56 |
| 28 | 67 | M | 1997 | – | 1:2048 | NA | 5.2 | 208 |
| 29 | 59 | M | 1997 | – | 1:4096 | NA | 6.9 | 166 |

[a]Summary of all target genes used in the study: PCR, polymerase chain reaction; IFA, immunofluorescent assay; WBC, white blood cells; +, positive; –, negative; M, male; F, female; NA, not available.

included fever, headache, chills, weakness, nausea, vomiting, diarrhea, abdominal pain, dizziness, dyspnea, cough, sore throat, stiff neck and cutaneous rash (Table 3). Fever ranged from 37.9°C to 40.6°C (mean ± SD: 39.4°C ± 0.8). The most frequent symptoms besides fever were headache, myalgia or arthralgia, chills, weakness, and nausea. Coexisting conditions were found in three patients and included inflammatory bowel disease, adult onset diabetes mellitus, and coronary artery disease, status post coronary artery bypass grafting.

Hemoglobin values in all patients ranged from 102 to 169 gm/L (mean ± SD: 136 ±1.7 gm/L). Leukopenia (defined as leukocyte count [WBC] <4.5 cells x $10^9$/L) was present in 15 (60%). Of 25 cases in which WBC was analyzed, the overall range was from 1.2 to 10.0 x $10^9$ cells/L (mean ± SD: 4.6 ± 2.3 x $10^9$ cells/L). Of 23 patients in whom platelet counts were analyzed, the overall range was from 36 to 397 x $10^9$ cells /L (mean ± SD: 153.3 ± 95 x $10^9$ cells/L). Both thrombocytopenia and leukopenia were present in 11 patients (48%). Thrombocytopenia was observed in 13 (57%). Serum aspartate aminotransferase (AST) levels were determined in 21 patients and ranged from 18 to 538 U/L (mean ± SD: 124.1 ± 146.9 U/L). AST levels were elevated in 11 patients (52%). Serial blood cell counts were available in six patients, and all showed WBC returning to normal values from 7 to 21 days after the illness started. Lymphopenia was usually seen during the acute phase of the disease (both relative and absolute) and was replaced by relative and then absolute lymphocytosis, beginning at day 9 and occurring up until day 21 in some cases.

Altogether, 26 case-patients (90%) had serum antibodies detected by IFA. The three case-patients that were IFA negative were positive by PCR, and no convalescent-phase sample could be obtained from these patients. In fact, acute- and convalescent-phase samples were obtained in 13 patients from the case-patient group. Seroconversion (defined as a fourfold rise in end-point titers in acute- and convalescent-phase samples) was demonstrated in seven case-patients. The remaining case-patients, whose condition was diagnosed by IFA, had elevated titers in the acute-phase sample, and the titers rose slightly or remained stable in the convalescent-phase sample (Table 2). The geometric mean titer in the acute-phase samples was 512 and 633.7 in the convalescent-phase samples. The interval between acute- and convalescent-phase serum samples

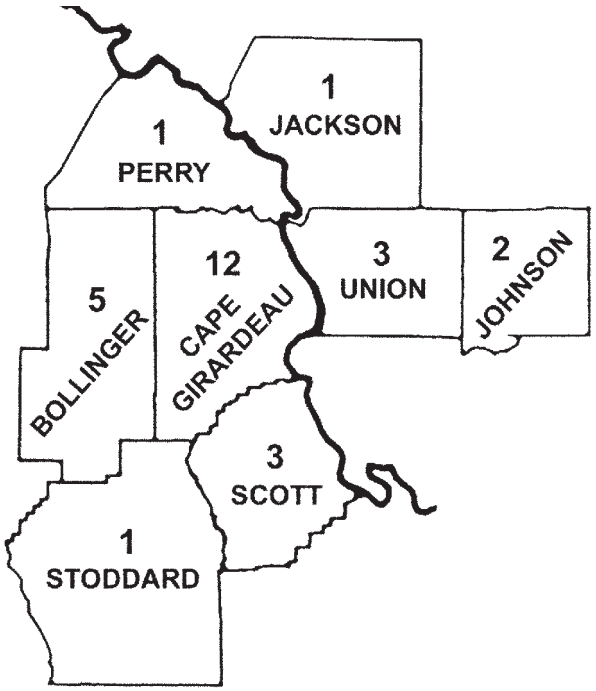


Figure. Counties in southeastern Missouri and southwestern Illinois in which cases of human monocytotropic ehrlichiosis (HME) were diagnosed from 1997 to 1999. Numbers represent HME cases in each county. A single case that occurred in Phelps County (south-central Missouri) is not shown.

ranged from 2 to 8 weeks. In the acute-phase serum samples, nine patients (31%) had titers of <1:64, four (14%) had titers between 1:64 and 1:128, seven (24%) had titers between 1:256 and 1:512, and nine (31%) had titers ≥1:1024. Of the convalescent samples, two (15%) had titers between 1:64 and 1:128, five (39%) between 1:256 and 1:512, and six (46%) ≥1:1024. Cross-reactive antibodies against *A. phagocytophilum* were found in nine cases (31%), and all end-point titers were 1:160 or less. In all of these cases, the IFA end-point titers against *E. chaffeensis* were 1:512 or greater.

Ehrlichial DNA was amplified by PCR in 15 of the 29 confirmed and probable cases and in 14 of the 25 confirmed cases (sensitivity: 52% and 56%, respectively). Of the 14 HME patients who tested negative by PCR, 10 (71.4%) had IFA titers ≥1:256 (eight of these case-patients had titers ≥1:1024). Of the 15 cases diagnosed by PCR, ehrlichial DNA was amplified in nine cases from one target gene, in four from two target genes and in two from all three target genes used in the study. Twelve cases were diagnosed by both PCR and IFA. No ehrlichial DNA was amplified from acute-stage blood specimens of the 20 patients in the nonseroconversion control group (specificity >95%). PCR testing confirmed the infection in all but one of the patients who seroconverted (sensitivity: 84%).

The positive likelihood ratio for PCR was theoretically infinite since the specificity in our study was 100%. However, because of the relatively small number of cases, a specificity of >95% seems more adequate. In a hypothetical situation of one false-positive PCR result in 100 tests performed, the positive likelihood ratio would have been 56 and 84 for sensitivity values of 56% and 84%, respectively. The negative likelihood ratio was 0.44 for a sensitivity value of 56% and 0.16 for a value of 84%. The posttest probabilities for a positive PCR test were 97% and 96% for sensitivity values of 84% and 56%, respectively. The posttest probabilities of a negative PCR test were 4.3% and 11.1% for sensitivities of 84% and 56%, respectively. Posttest probabilities were calculated on the basis of the incidence of HME in the total population of the study (102 patients), that is, patients who met the case definition used in this study.

DNA sequencing analysis of PCR products was performed on samples from five patients that yielded PCR products for the 16S rRNA, *nadA* and 120-kDa protein genes. The sequences revealed greater than 99% homology with the published sequences of *E. chaffeensis* genes.

*Ehrlichia chaffeensis* was not cultivated from any of the blood samples that were shipped from Missouri to Texas.

Twelve (41%) of the HME patients required hospitalization: eight men and four women. Differences in age and laboratory data between hospitalized and nonhospitalized patients were not statistically significant, except for the degree of thrombocytopenia (Table 4).

All patients in whom HME was diagnosed were treated with doxycycline. Duration of treatment ranged from 2 to 4 weeks. Fever resolved within 24 hours in three patients (19%), within 48 hours in 10 patients (62%), and within 72 hours in three patients (19%).

Comparison of clinical parameters between HME case-patients and the control group showed no statistically significant differences between the two groups, except for the presence of cough in the HME case-patient group, illustrating again the nonspecific clinical presentation of this disease (Table 3). However, statistically significant differences between the two groups were observed for age, WBC count, and absolute neutrophil count, but not for platelet count, absolute lymphocyte count, or aspartate aminotransferase levels (Table 3).

## Discussion

HME is a prevalent disease in southeast Missouri, an area similar to most of the rural southeastern United States in terms of its white-tailed deer-lone star tick zoonotic cycle of *E. chaffeensis* and exposure to the bite of infected ticks. We enrolled 102 patients in the 3-year study, and 29 (28.4%) of patients had either definite or probable HME. For 1997, 1998, and 1999, the calculated incidence for HME was 2, 4.7, and 3 per 100,000 population, respect-

Table 3. Comparison of selected clinical features and laboratory data between patients with human monocytotropic ehrlichiosis (HME) (case-patient group) and noncase group (control group). Cape Girardeau, Missouri, 1997–1999

| Clinical Feature | HME case-patient group N (%) | Control group N (%) | p value |
|---|---|---|---|
| **Fever** | 29 (100) | 20 (100) | NA |
| Headache | 21 (72) | 14 (70) | 0.89[a] |
| Dizziness | 6 (21) | 2 (7) | 0.44[b] |
| Myalgia/arthralgia | 20 (69) | 10 (50) | 0.29[a] |
| Chills | 13 (45) | 7 (35) | 0.69[a] |
| Weakness | 11 (38) | 3 (15) | 0.15[a] |
| Nausea | 11 (38) | 3 (15) | 0.13[a] |
| Vomiting | 2 (7) | 2 (10) | 1.00[b] |
| Diarrhea | 3 (10) | 2 (10) | 1.00[b] |
| Abdominal pain | 2 (7) | 1 (5) | 1.00[b] |
| Cough | 7 (24) | 0 (0) | 0.03[b] |
| Sore throat | 6 (21) | 0 (0) | 0.07[b] |
| Rash | 6 (21) | 0 (0) | 0.07[b] |
| Stiff neck | 6 (21) | 0 (0) | 0.07[b] |
| Confusion | 2 (7) | 0 (0) | 0.50[b] |
| **Laboratory** | Mean ± SD/median | Mean ± SD/median | p value |
| Age | 48.6 ± 17.5 | 35.7 ± 19.9 | 0.02[c] |
| Leukocytes x $10^9$ cells/L | 4.67 | 6.25 | 0.04[d] |
| Neutrophils x 109 cells/L | 2645 | 3810 | 0.03[d] |
| Lymphocytes x $10^9$ cells/L | 1677 | 1897 | 0.36[d] |
| Platelets x $10^9$ cells/L | 172± 101.8 | 250.8 ± 137.5 | 0.06[c] |
| Aspartate aminotransferase (U/L) | 63 | 32 | 0.84[d] |

[a]Calculated by using Fisher exact test.
[b]Calculated by using chi-square test.
[c]Calculated by using t-test.
[d]Calculated by using Mann-Whitney rank sum test.

ively (incidence calculations were based on the total population of all counties where the patients lived. Population figures were obtained from the U.S. Census Bureau Web site and are based on the 2000 U.S. Census (URL: http://quickfacts.census.gov/qfd/states/29000.html). These incidence figures are higher than expected, even for an HME-endemic area such as Missouri. On the other hand, HME has probably been underestimated throughout the rural southeastern and south central states. In this particular disease-endemic area, our case-patients were identified mainly in one primary care-based physician's office that cares for a population base of approximately 7,000 persons. Therefore, the real incidence of HME is likely higher in Cape Girardeau and surrounding counties than this overall study dictated. Physicians who diligently pursue the diagnosis are likely to be surprised by the frequency with which cases are identified. In fact, Carpenter et al. (21) reported a higher than expected incidence of HME in a prospective study performed in central North Carolina, an area well known for a high incidence of Rocky Mountain spotted fever.

Our clinical case definition was broad and tried to include all potential cases of HME in the disease-endemic area. Our laboratory criteria to diagnose HME in this study are patterned after those of the Council of State and Territorial Epidemiologists (CSTE), although our criteria are even more stringent regarding PCR interpretation (24). We required the amplification of ehrlichial DNA by two primer sets or confirmation of PCR results by two different laboratories. We also required positive serologic assays, along with the PCR results, to confirm a suspected case. Our aim was to avoid the inclusion of cases in which PCR might have amplified ehrlichial DNA nonspecifically. However, our specificity for PCR testing was 100%.

Our serologic criteria for laboratory diagnosis of HME are the same as those proposed by CSTE. For confirmation purposes, we considered end-point titers of 1:256 or greater as a criterion when only one serum sample was available for diagnosis. IFA seroconversion has been considered the standard criterion for the diagnosis of HME. However, samples with high end-point titers by IFA (≥1:256) are highly suggestive of acute HME unless the patient is recovering from an acute infection and the titers are returning to normal levels. High end-point titers usually return to lower levels several months after the patient recovers clinically. In three of our case-patients, antibodies against *E. chaffeensis* were still detectable 8 to10 months after infection. In these cases, PCR or rising IFA titers would help solve the diagnostic dilemma. Frequently, diagnostic IFA end-point titers were lacking at the time of the patient's first visit. In this series, 31% of acute-phase serum samples had a diagnostic titer. In addition, 45% of the samples that tested positive (≥1:64) at the initial visit had titers <1:256. Therefore, convalescent-phase samples are highly desirable to confirm cases of HME reliably. Another important finding is the presence of cross-reacting

Table 4. Association of selected demographic variables and laboratory data with severity of illness for 29 patients with human monocytotropic ehrlichiosis (HME), Cape Girardeau, Missouri, 1997–1999

| Parameter | Nonhospitalized mean ± SD/Median | Hospitalized mean ± SD/median | p value |
|---|---|---|---|
| Age (y) | 45.8 ± 17.5 | 51.8 ± 19.1 | 0.41 |
| Leukocyte count, x $10^9$ L | 5.1 ± 2.6 | 4.0 ± 1.7 | 0.21 |
| Platelets | 192.4 ± $10^9$ | 117 ± 66 | 0.05 |
| Neutrophil counts, x $10^9$ [a] | 2,960 | 2,590 | 0.59 |
| Lymphocyte counts x $10^9$ L | 1,948.5 ± 112.7 | 1,383.4 ± 1,167.7 | 0.26 |
| Aspartate aminotransferase, U/L[a] | 89 | 49 | 0.96 |

[a]Differences analyzed by Mann-Whitney rank sum test. All others analyzed by t-test.

antibodies against *A. phagocytophilum* in 31% of our patients. In these patients, the titers against *E. chaffeensis* were higher than the titers against *A. phagocytophilum*, and according to published criteria, these cases most likely represent HME instead of *A. phagocytophilum* infections (25). In addition, PCR testing did not detect *A. phagocytophilum* DNA in any of the patients' blood samples. The proportion of patients with cross-reacting antibodies is higher than reported in other series, and at this time we do not know the reason for this finding (6,26).

*E. ewingii* infections likely occur in this patient population as well. The specificity of the PCR primer sequences ensure that none of the patients with infections diagnosed by PCR amplification had *E. ewingii* infection. In addition, we were able to test the 49 case-patients included in the final analysis of the study retrospectively. After the first reports of *E. ewingii* cases in humans in 1999, we retrieved DNA from our freezers from those 49 case-patients. *E. ewingii*–specific primers were used and no amplicons were obtained. The possibility that a serum specimen that contained antibodies stimulated by *E. ewingii* might have been labeled as indicating HME cannot be excluded, owing to cross-reactivity with *E. chaffeensis*.

The sensitivity of PCR was calculated on the basis of the total number of cases diagnosed by IFA. The relatively low sensitivity (56%) in our study when compared to that of Everett et al. (87%) and Standaert et al. (100%) is noteworthy. We do not have a clear explanation for this difference. However, in those series all patients in whom ehrlichial DNA was amplified from blood had low or negative IFA titers in the acute-phase serum sample, whereas in our series a substantial number of patients had acute-phase serum samples with high IFA end-point titers. This difference suggests that the ehrlichemia might be lower in cases where the immune response is well established. In fact, a t-test analysis of the geometric mean titer of PCR-positive versus PCR-negative persons yielded a statistically significant difference ($p < <0.007$), suggesting that seropositive patients are less likely to be PCR-positive. PCR sensitivity increased to 84% when only cases diagnosed by seroconversion were used to calculate it. The specificity of PCR was 100%.The positive and negative likelihood ratios and posttest probabilities based on sensitivity and specificity suggest that PCR is a useful tool for diagnosing HME in the early phase of the disease.

Our failure to isolate *E. chaffeensis* from these cases is most likely related to the delay in inoculating the blood samples of patients with HME into cell culture. The interval between blood sampling and inoculation may play a critical role when attempts to obtain isolates of *E. chaffeensis* are made (22).

The spectrum of illness in our study ranged from mild to life-threatening disease that required hospitalization and intensive care; 41% of the patients in our study were hospitalized. Since we detected cases based on a clinical definition that included fever for ≥3 days, we probably excluded the mildest cases of the disease in which a self-limited illness developed, which resolved spontaneously. In fact, asymptomatic seroconversion has been documented in soldiers who underwent field training and were exposed to ticks. However, whether the antigenic stimulation in those cases was actually triggered by *E. chaffeensis* or by some other antigenically related, less pathogenic bacterium, such as *E. ewingii* or the unnamed white-tailed deer *Anaplasma* species (both also associated with the lone star tick) is not known (18,27). The clinical syndrome of HME observed in this study is similar to that described in other series (7,8,10,11,20–22) in that it can be a serious illness that requires hospitalization in a large number of cases, even though the prospective, clinic-based nature of the study allowed more mild cases to be identified earlier in the course of illness.

Comparison of the case-patient group and the control group revealed the important difficulty in clinical diagnosis: few clinical symptoms differed between case-patients and non–case-patients. Even the signs and symptoms that showed some differences are nonspecific and can occur in other clinical conditions. The relatively high frequency of neurologic and respiratory signs is noteworthy, showing the potential severity of this disease. Among the few patients that underwent lumbar puncture in this study, the CSF showed pleocytosis with lymphocytic predominance (data not shown). Age, white blood cell counts, and absolute neutrophil counts were statistically significantly different between the HME and non-HME patients; thrombocytopenia was nearly statistically different ($p = 0.06$),

pointing out again the importance of leukopenia and thrombocytopenia as diagnostic clues during the acute phase of the disease. The differences in age of the patients confirm once again that HME tends to affect older people more frequently than younger people (*E. chaffeensis* infection also may cause a milder illness in the young).

In summary, HME is an emerging tick-borne disease; its epidemiology and clinical spectrum are still being determined, and the incidence is higher than previously thought. The clinical diagnosis is challenging, and a high degree of suspicion is required to order specific diagnostic tests to confirm the diagnosis. PCR appears to be a useful diagnostic test during the early phase of this potentially life-threatening tick-borne zoonosis.

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Dr. Olano, an assistant professor at the University of Texas Medical Branch at Galveston, is engaged in research on pathogenesis and immunity in rickettsial and ehrlichial infections. Dr. Masters, a family physician in Cape Girardeau, Missouri, has investigated *Amblyomma americanum*–associated erythema migrans for many years.

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Address for correspondence: Juan P. Olano, Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0747, USA; fax: 409-772-2500; email: jolano@utmb.edu

# *Mycobacterium abscessus* and Children with Cystic Fibrosis

**Isabelle Sermet-Gaudelus,* Muriel Le Bourgeois,* Catherine Pierre-Audigier,* Catherine Offredo,* Didier Guillemot,† Sophie Halley,* Chantal Akoua-Koffi,* Véronique Vincent,† Valérie Sivadon-Tardy,‡ Agnès Ferroni,* Patrick Berche,* Pierre Scheinmann,* Gérard Lenoir,* and Jean-Louis Gaillard‡**

We prospectively studied 298 patients with cystic fibrosis (mean age 11.3 years; range 2 months to 32 years; sex ratio, 0.47) for nontuberculous mycobacteria in respiratory samples from January 1, 1996, to December 31, 1999. *Mycobacterium abscessus* was by far the most prevalent nontuberculous mycobacterium: 15 patients (6 male, 9 female; mean age 11.9 years; range 2.5–22 years) had at least one positive sample for this microorganism (versus 6 patients positive for *M. avium* complex), including 10 with >3 positive samples (versus 3 patients for *M. avium* complex). The *M. abscessus* isolates from 14 patients were typed by pulsed-field gel electrophoresis: each of the 14 patients harbored a unique strain, ruling out a common environmental reservoir or person-to-person transmission. Water samples collected in the cystic fibrosis center were negative for *M. abscessus*. This major mycobacterial pathogen in children and teenagers with cystic fibrosis does not appear to be acquired nosocomially.

Since 1990, an increasing number of studies have reported the recovery of nontuberculous mycobacteria from the respiratory tract of patients with cystic fibrosis (1–4). *Mycobacterium abscessus* (formerly *M. chelonae* subsp. *abscessus*), a rapidly growing mycobacterium of the *M. fortuitum* complex, is of particular concern. It can cause severe lung disease, which spontaneously advances until it becomes debilitating or fatal (5,6). It may be responsible for disseminated infections in patients undergoing lung transplantation (7). This organism is usually also susceptible to only a few drugs (8), and some strains may exhibit multidrug resistance (7).

The frequency of isolation of *M. abscessus* in cystic fibrosis patients is unclear. Many studies on nontuberculous mycobacteria in such patients did not distinguish *M. abscessus* and *M. chelonae*, formerly two subspecies of *M. chelonae*, and used the designation *M. chelonae–M. abscessus*, *M. chelonae* group, or even *M. fortuitum* complex. Moreover, most studies were conducted with adults (1,2,4). How cystic fibrosis patients become contaminated is also poorly understood. *M. abscessus* has been reported to be acquired iatrogenically in non–cystic fibrosis patients (9). The members of the *M. fortuitum* complex are saprophytic organisms living in soil and water that are ubiquitous in hospital environments and survive well in adverse conditions (10–13). Aerosols, pulmonary function equipment, and bronchoscopes are thus potential sources of contamination for patients with cystic fibrosis. Alternatively, transmission from patients to patients attending the same department-care facilities might occur, although this finding has been recently challenged (14).

We encountered one case of *M. abscessus* infection in a patient with cystic fibrosis in 1995. The recovery of this unusual pathogen prompted us to prospectively evaluate the rate of isolation of *M. abscessus* in the cystic fibrosis patients attending our center, the degree of transmissibility of this organism, and its clonality, by using DNA-based identification and typing systems.

## Patients and Methods

### Description of Study

All patients with cystic fibrosis who attended the pediatric department of Necker Hospital for Sick Children from January 1, 1996, to December 31, 1999, provided at least one sputum sample per year, which was processed for the culture of acid-fast bacilli (AFB). Patients who provided a positive sample then submitted ≥3 sputum samples for AFB smear and culture over the next 3 months. AFB smears and cultures were checked quarterly thereafter.

*Hôpital Necker-Enfants Malades, Assistance Publique–Hôpitaux de Paris, Paris, France; †Institut Pasteur, Paris, France; and ‡Hôpital Raymond Poincaré, Assistance Publique–Hôpitaux de Paris, Garches, France

### Cultures of Respiratory Specimens

Specimens were decontaminated with NALC-NaOH-oxalic acid (0.25% *N*-acetyl-L-cysteine–1% sodium hydroxide–5% oxalic acid) (15). AFB smears were stained with auramine-rhodamine and scored as previously described (16). Two Löwenstein-Jensen slants were inoculated for each specimen, one of which was incubated at 37°C and the other at 30°C. The slants were examined twice weekly for 2 weeks and then weekly for a further 10 weeks.

### Environmental Samples

Water samples taken from the hospital's hot and cold water supply systems were collected in sterile plastic bottles. Samples (100 mL) were decontaminated with 1% NaOH without prior concentration by filtration (17). The inner surfaces of respiratory devices (e.g., nebulizers, bronchoscopes) were rinsed with 1 to 10 mL of sterile distilled water; the water used for rinsing was processed for the culture of AFB without prior decontamination with 1% NaOH.

### Species Identification

Rapidly growing mycobacteria recovered from clinical and environmental samples were identified by standard techniques (17) and *hsp65* sequencing (18). The *M. avium* complex was identified by the AccuProbe technique (Gen-Probe Inc., San Diego, CA). The *hsp65* genomovars of *M. abscessus* were referred to as T (identical to the type-strain *M. abscessus* ATCC 19977$^T$), -5a (differing from ATCC 19977$^T$ by 5 nt, and identical to the reference strain *M. abscessus* IP970272), -5b (differing from ATCC 19977$^T$ by 5 nt, and identical to the reference strain *M. abscessus* IP970453), and –6 (differing from ATCC 19977$^T$ by 6 nt, and identical to the reference strain *M. abscessus* IP140420009), as previously described (18).

### PFGE Analysis

*M. abscessus* isolates were analyzed by PFGE as described by Wallace et al., with minor modifications (9). Restriction fragments obtained after digestion with *Dra*I and *Xba*I were separated in 0.5 x TBE buffer (0.025 M Tris, 0.5 mM EDTA, and 0.025 M boric acid) supplemented with 50 µM thiourea (19), using a CHEF-DR III system (Bio-Rad, Richmond, CA) at 14°C and 6 V/cm$^2$. Pulse times were ramped linearly from 1.5 to 21.5 s for 23 h. A size standard (bacteriophage ? concatemers) was run in parallel in each experiment. Restriction patterns were analyzed with the Taxotron package (Taxolab Software, Institut Pasteur, Paris, France) comprising the RestrictoScan, RestrictoTyper, Adanson, and Dendrograph programs.

## Results

### Screening the Study Population for Nontuberculous Mycobacteria

A total of 298 patients with cystic fibrosis (1,525 sputum samples; mean of 5.0 samples per patient) followed up at our institution were screened for *M. abscessus* from January l, 1996, to December 31, 1999. The age of the patients ranged from 2 months to 32 years (mean 11.3 years). The sex ratio was 0.47 (140 male/158 female patients). Samples from two patients could not be analyzed because the samples were repeatedly contaminated (<1 interpretable culture per year during the study period). Of the 296 patients with interpretable cultures, 29 (9.80%) provided at least one sample positive for nontuberculous mycobacteria. Twelve of the 296 patients had *M. abscessus* alone, 3 had *M. abscessus* and *M. gordonae*, 6 had *M. avium* complex, 4 had *M. gordonae*, 1 had *M. fortuitum*, 1 had *M. kansasii*, and 2 had organisms not related to any known species. Thirteen patients provided at least three positive samples, 10 involving *M. abscessus* and 3 *M. avium* complex. Two of these patients were twin sisters, one colonized with *M. abscessus* (patient no. 5) and the other with *M. avium* complex.

### Description of Cases with *M. abscessus* Isolation

Fifteen (5%) of the 296 patients with interpretable cultures provided at least one sample positive for *M. abscessus*. Ten of these patients had ≥3 positive samples, including six with positive AFB smears (Table). Mycobacterial disease was documented in four patients: a 16-year-old boy (patient no. 4) with parenchymal condensation of the left lower lobe on chest x-ray and computed tomographic (CT) scan, which disappeared only under anti–*M. abscessus* treatment; a 10-year-old boy (patient no. 6), whose rapidly deteriorating and ultimately fatal condiction was associated with diffuse bronchiectasis on CT scan; a 9-year-old girl (patient no. 7), who had a massive, granulomatous pneumonia of the right lung that led to pneumonectomy, and who died after 15 months of bacteriologically ineffective anti–*M. abscessus* treatment; and a 2-1/2-year-old girl (patient no. 8) with segmental condensation of the right mid-lobe on chest x-ray and CT scan, which disappeared only under anti–*M. abscessus* treatment.

All but one (patient no. 1) of the 15 patients were recognized during the study period. Some of the patients who were identified in the first year may have previously gone undetected, as nontuberculous mycobacteria had not been sought before (patients nos. 2, 3, 4, 5, and 7). The *M. abscessus* isolates belonged to genomovars T, -5a, and -6,

Table. Chronology of case-patients with *Mycobacterium abscessus* isolation

| Case no. | Age (y)/sex | Date of first isolation | No. of pos. cultures/total AFB cultures[b] | No. of pos. AFB smears/total AFB smears[b,c] | | *hsp65* genomovar[d] | Sputum microbiologic results[e] |
|---|---|---|---|---|---|---|---|
| 1 | 2.5 / M | Jan 1996 | 37/48 | 19/31 | (+++) | -6 | Negative |
| 2 | 13 / F | Feb 1996 | 2/14 | 0/9 | | T | *Pseudomonas aeruginosa* |
| 3 | 15 / F | Apr 1996 | 3/41 | 0/21 | | -5a | *P. aeruginosa* |
| 4 | 16 / M | Apr 1996 | 5/8 | 1/6 | (+) | T | *P. aeruginosa*, *Staphylococcus aureus*, *Aspergillus fumigatus* |
| 5 | 14 / F | May 1996 | 8/11 | 4/8 | (++) | T | *P. aeruginosa*, *S. aureus, A. fumigatus* |
| 6 | 10 / M | May 1996 | 4/25 | 0/12 | | T | *P. aeruginosa, Alcaligenes xylosoxidans* |
| 7 | 9 / F | May 1996 | 8/8 | 4/7 | (+++) | T | *P. aeruginosa*, *Aspergillus fumigatus* |
| 8 | 2.5 / F | Nov 1997 | 4/6 | 2/5 | (++) | -5a | Negative |
| 9 | 7 / F | July 1998 | 2/24 | 0/7 | | -5a | *S. aureus* |
| 10 | 17 / M | Sept 1998 | 3/9 | 0/6 | | T | *S. aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *A. fumigatus* |
| 11 | 8 / F | Sept 1998 | 3/12 | 0/4 | | T | *P. aeruginosa*, *Staphylococcus aureus* |
| 12 | 18/F | July 1999 | 1/5 | 0/3 | | T | *P. aeruginosa*, *A. fumigatus* |
| 13 | 9/M | Sept 1999 | 1/6 | 0/3 | | -6 | *S. aureus* |
| 14 | 16/M | Oct 1999 | 5/13 | 4/6 | (+) | -5a | *S. aureus*, *A. fumigatus* |
| 15 | 22/F | Nov 1999 | 1/6 | 0/3 | | -6 | *P. aeruginosa*, *S. aureus* |

[a]AFB, acid-fast bacilli.
[b]Samples obtained from patients from January 1996 to December 2000; only samples obtained before the administration of antimycobacterial drugs are considered in treated patients.
[c]Symbols in parentheses: AFB density.
[d]See Methods.
[e]Organisms recovered from at least three sputum samples within the 12 months before the first isolation of *M. abscessus*.

with a slightly higher prevalence of genomovar T (Table). Genomovar T was involved in the two fatal cases recorded during the study period (patients nos. 6 and 7).

### Characteristics of Patients Positive for *M. abscessus*

The 15 patients positive for *M. abscessus* were predominantly females (sex ratio, 0.40). Their mean age at the time of the first culture positive for *M. abscessus* (11.9 years, range 2.5–22 years) was very similar to the mean age of the entire study population. However, the mean age was lower than that for patients positive for *M. avium* complex (17.5 years; range 13–25 years). Of the 14 patients who underwent genotype analysis, 8 were homozygous for deletion of the phenylalanine in position 508, and 4 were heterozygotous for this deletion plus another mutation. Pulmonary function at the time of the first isolation of *M. abscessus* was highly variable, with forced expiratory volume in 1 second and forced vital capacity values ranging from 14% to 99%, and 31% to 104% of predicted values, respectively. Schwachman score (20) also greatly varied among patients (range 40–85). The most prevalent associated disorders included bronchiectasis (13 cases), gastroesophageal reflux (3 cases), and allergic bronchopulmonary aspergillosis (3 cases). All patients had pancreatic insufficiency. Nine of the 15 patients were colonized (at least three positive sputum samples within the previous 12 months) with *Pseudomonas aeruginosa*. None was colonized with *Burkholderia cepacia*.

We analyzed records of all treatments received by the patients within the 12 months preceding the first isolation of *M. abscessus*, including therapeutic aerosols. All of the patients had received IV antibiotics (1–5 two-week IV courses; median 3 courses), combined with aerosol antibiotics at home in 11 patients (tobramycin, 3 patients; colistin, 8 patients). Six patients had received aerosolized deoxyribonuclease. Two patients had received oral corticosteroids, and four had received inhaled corticosteroids.

### Environmental Study

A total of 93 water samples collected from 40 water supply points in the cystic fibrosis center were studied. Three samples (3.2%) from two water supply points tested positive for rapidly growing mycobacteria (*M. mucogenicum,* two samples; *M. peregrinum*: one sample). None of the samples tested positive for *M. abscessus*. None of the 12 respiratory devices (3 bronchoscopes, 9 nebulizers) studied in October 1997 tested positive for any nontuberculous mycobacteria.

### PFGE Analysis of *M. abscessus* Isolates

PFGE was used to compare the isolates from 14 patients positive for *M. abscessus* (the isolates from patient no. 7 could not be subcultured for testing because of inadequate storage). We studied all isolates from each patient who provided ≤3 positive cultures and a maximum of five isolates from each patient with >3 isolates. The isolates from three patients (patients nos. 6, 11, and 12) gave unreadable restriction patterns with classical protocols, despite multiple attempts. This phenomenon is common with mycobacteria, particularly *M. abscessus* (9) and is probably related to Tris-dependent site-specific cleavage of the DNA (19). Nondegradative PFGE was only achieved by running gels in the presence of thiourea, which has been shown to protect the DNA from strand cleavage (21). We were therefore able to type all isolates from the 14 patients by PFGE. Each of these 14 patients had isolates of a unique genotype that was unrelated to the genotype of any other patient (Figure). No differences were detected between isolates from the same patient (data not shown), even if the interval between the first and last isolation was as long as 4 years (patient no. 1).

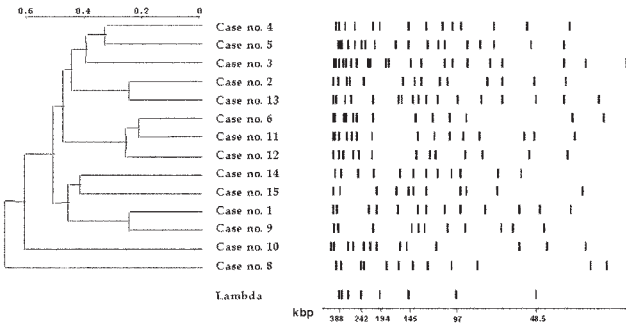


Figure. Pulsed-field gel electrophoresis analysis of *Dra*I-digested DNA from *Mycobacterium abscessus* isolates. Restriction patterns of isolates from 14 patients are shown, with a dendrogram of similarity; λ concatemers were used as size standards.

## Discussion

*M. abscessus* was the predominant nontuberculous mycobacterium recovered from the patients attending our center. Approximately 5% of the patients we screened provided at least one sputum sample positive for this organism, and >65% of these patients had ≥3 positive samples. Other rapidly growing mycobacteria were far behind (*M. fortuitum*, one patient). This finding confirms that *M. abscessus* differs from *M. chelonae* and from other members of the *M. fortuitum* complex by its particular propensity to cause lung disease in a variety of clinical settings. In a series of 154 cases of lung infection caused by rapidly growing mycobacterium in patients with and without cystic fibrosis, >80% of isolates were *M. abscessus*; *M. fortuitum* was isolated in <15% and *M. chelonae* in <1% of patients (22).

In contrast with other studies on cystic fibrosis populations composed of teenagers and adults (2,4), we found that the *M. avium* complex was isolated less frequently, with an overall prevalence (percentage of patients with at least one positive nontuberculous mycobacterial culture) of approximately 2%. Other pediatric cystic fibrosis centers have reported similar findings (23,24). This finding suggests that *M. abscessus* is the most prevalent mycobacterial pathogen in children and teenagers with cystic fibrosis. This finding is further supported by the lack of cases involving *M. avium* complex in patients <13 years of age. However, this finding does not preclude epidemiologic variations between countries or institutions.

Nosocomial acquisition of *M. abscessus* has been well documented in patients without cystic fibrosis. Several nosocomial outbreaks of infection or pseudoinfection have been attributed to this organism after cardiac surgery, bacteremia associated with hemodialysis, and pseudoinfections due to contaminated bronchoscopes (9). Epidemiologic investigations showed that these outbreaks resulted from the use of contaminated water. Disinfectants may be ineffective against *M. abscessus* in real conditions of use (12). PFGE has been used to retrospectively analyze the clinical and environmental isolates recovered during *M. abscessus* outbreaks (9). Each of the outbreaks with typeable isolates clearly involved a single strain, which was usually recovered from the water supply system.

Our results do not support nosocomial acquisition of *M. abscessus*. First, the recovery rate of this organism remained constantly low throughout the study. Previous nosocomial outbreaks involving patients without cystic fibrosis were characterized by much higher attack rates. Second, we did not find any link between the use of respiratory devices and the acquisition of *M. abscessus*. Patients positive for *M. abscessus* did not receive more aerosol treatments than did patients with similar clinical status (not shown). The patients used their personal nebulizer at the center and received aerosols in their own rooms. Sterile saline was used when the aerosol was mixed. Reusable respiratory devices were disinfected according to validated protocols and were washed exclusively with sterile water. During the study period, no patients without cystic fibrosis, even severely immunocompromised ones, were infected with *M. abscessus* as a result of a contaminated bronchoscope in our pediatric department. Third, although various rapidly growing mycobacteria were recovered from several water supply points in our center, *M. abscessus* was not isolated. Finally, PFGE analysis demonstrated that the cases involved unrelated strains, which argues against a common

source of contamination or patient-to-patient transmission. Similar results have been recently reported with fewer patients (14). The low transmissibility, if any, of *M. abscessus* from person to person is further supported by the observation of twin sisters in our series, only one of whom was colonized with *M. abscessus*.

Whether specific measures are necessary to prevent *M. abscessus* infection in patients with cystic fibrosis is questionable (14). Our epidemiologic results indicate that few potential control approaches exist. A strict segregation policy seems unnecessary because apparently no risk of person-to-person transmission of *M. abscessus* exists (14, this study). Further epidemiologic studies are required before recommendations for infection-control precautions can be formulated.

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Dr. Sermet-Gaudelus is a physician in the Pediatric Department of the Necker Hospital for Sick Children, where she is studying a cohort of 350 cystic fibrosis patients. She is especially interested in emerging bacterial and fungal agents in such patients. She is now finishing a doctoral thesis on the electrophysiology of the nasal mucosa in cystic fibrosis patients.

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Address for correspondence: Jean-Louis Gaillard, Laboratoire de Microbiologie, Hôpital Raymond Poincaré, 104 Boulevard Raymond Poincaré, 92380, Garches, France; fax: +33 147 10 79 49; email: jean-louis.gaillard@rpc.ap-hop-paris.fr

# The Rabbit as a New Reservoir Host of Enterohemorrhagic *Escherichia coli*

**Alexis García* and James G. Fox***

We investigated the prevalence of enterohemorrhagic *Escherichia coli* (EHEC) in rabbits acquired from two commercial vendors and a local petting zoo. Fecal samples from 34 Dutch Belted (DB) and 15 New Zealand White (NZW) rabbits were cultured; and isolates were biotyped, serotyped, tested by polymerase chain reaction (PCR), and genotyped by repetitive-element sequence–based PCR (Rep-PCR). Seven (25%) of 28 DB rabbits acquired from one commercial source were positive for EHEC, including O153:H- and O153:H7. One (9%) of 11 NZW rabbits from the same source was positive for *eae*-, *stx*1+ O153 strains. In contrast, six DB rabbits from another commercial source and four rabbits from a petting zoo were negative for EHEC. Rep-PCR demonstrated that the O153 EHEC and O145 enteropathogenic *E. coli* were two distinct clones. Our study indicates that rabbits are a new reservoir host of EHEC that may pose a zoonotic risk for humans.

*Escherichia coli* O157:H7 is a leading foodborne enteric pathogen associated with human illness, including hemorrhagic colitis and hemolytic uremic syndrome (HUS), the leading cause of acute renal failure in children (1). Similarly, non-O157 enterohemorrhagic *E. coli* (EHEC) serotypes have been implicated in outbreaks of disease worldwide and are currently considered emerging pathogens by the World Health Organization (WHO) (2). Most EHEC infections in humans are foodborne, and the source of infection is an animal reservoir. Cattle and other ruminants are considered major reservoirs hosts of EHEC (3). Recent reports have also emphasized that farm animals and their environment pose a zoonotic risk for humans, based on outbreaks of *E. coli* O157:H7 infection among farm visitors (4).

Identifying bacterial pathogens in natural hosts is important because they constitute potential reservoirs for zoonotic transmission (5). We recently described an outbreak of hemorrhagic diarrhea and hemolytic uremic syndrome (HUS) in Dutch Belted (DB) rabbits naturally infected with EHEC O153:H- (6). In the current study, we investigated the prevalence of EHEC in laboratory rabbits acquired from two commercial vendors and in rabbits from a local petting zoo to assess their potential as reservoir hosts.

*Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

## Materials and Methods

### Rabbits

Fecal samples were collected from 34 DB and 15 New Zealand White (NZW) *Pasteurella multocida*–free laboratory rabbits acquired at various times from one commercial source. Fecal samples were also collected from six DB rabbits that were acquired from a second vendor and from four rabbits of various breeds that belonged to a local petting zoo.

### Bacterial Culture and Isolation

Fecal pellets were homogenized in tryptic soy broth (Remel, Lenexa, KS) and incubated at 37°C overnight. Each sample was then plated on Rainbow Agar O157 (Biolog, Hayward, CA) and incubated at 37°C for approximately 36 hours. Bacterial colonies were selected on the basis of color. Pink and purple colonies were selected and restreaked on MacConkey or blood agar plates (Remel) or both. All the *E. coli* isolates were confirmed biochemically and characterized by using API 20E strips (Biomerieux Vitek, Hazelwood, MO). Selected *E. coli* organisms were serotyped at the Pennsylvania State University *E. coli* Reference Center (ECRC).

### DNA Extraction and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from bacteria by using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA). PCR primers and conditions to detect *eae*, *stx1B*, and *stx2A* and variants have been described previously (6).

### Southern Blot Analysis

Southern blot analysis was performed by using a Shiga toxin 1B (*stx1B*) probe generated by PCR amplification of EHEC O153:H- DNA to confirm *stx1B* in EHEC strains isolated from rabbits. Fifteen microliters of amplicon underwent electrophroresis through a 1.3 % agarose gel and was transferred onto a Hybond N nylon membrane, as outlined by the manufacturer (Amersham, Piscataway, NJ). DNA was then cross-linked using the UV Stratalinker 1800 (Stratagene, La Jolla, CA). The fixed DNA was subsequently hybridized with the *stx1B* probe. The probe was labeled with horseradish peroxidase, exposed in the presence of luminol to Hyperfilm-ECL as outlined by the manufacturer (Amersham).

### High-Resolution Genotyping by Repetitive-Element Sequence-Based PCR (Rep-PCR)

To perform Rep-PCR chromosomal profiling, genomic DNA was extracted from bacteria by using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and quantified by using a GeneQuant *pro* (Biochrom Ltd., Cambridge, UK) spectrophotometer. Two primer pairs were used for the amplification reactions (7): REP1R-I (5′-IIIICGICGICATCIGGC-3′), REP2-I (5′-ICGICTTATCIGGCCTAC-3′); and ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′). PCR reaction mixtures were prepared by using puReTaq Ready-To-Go PCR Beads (Amersham) containing 50 pmol of each primer and 1 μL (100 ng) of DNA to a total volume of 25 μL. Rep-PCR reactions were performed by using a Techne Genius (Techne Inc., Princeton, NJ) thermal cycler with the following conditions: for the REP primers, initial denaturation (94°C, 7 min), followed by 30 cycles of denaturation (94°C, 30 s), annealing (40°C, 1 min), and extension (65°C, 8 min). A final extension (65°C, 16 min) completed the cycling protocol. For the ERIC primers, initial denaturation (95°C, 5 min) was followed by 35 cycles of denaturation (92°C, 45 s), annealing (51°C, 1 min), and extension (70°C, 10 min). A final extension (70°C, 20 min) completed the cycling protocol. PCR amplicons were visualized after electrophoresis in a 3% agarose gel and staining with ethidium bromide.

## Results

### Bacterial Cultures

Rabbit fecal samples cultured on Rainbow agar O157 yielded *E. coli* colonies that ranged in color from pink to purple. No black colonies consistent with *E. coli* O157 were observed (8).

Biochemical and molecular characterization of the rabbit *E. coli* strains is summarized in the Table. Most O153 strains were negative for rhamnose and sucrose. Some colonies of the O153:H- EHEC strain isolated from the first outbreak of HUS in DB rabbits (6) and a strain of unknown O serotype that showed autoagglutination were sorbitol negative like most O157:H7 EHEC organisms isolated from humans. In addition, two O145:H- EPEC were sorbitol negative.

### Prevalence of EHEC Strains

Seven (25%) of 28 DB rabbits acquired from one commercial source were positive for *eae*+, *stx1*+ *E. coli,* including serotypes O153:H- and O153:H7. One (14%) of the 7 rabbits was infected with an EHEC of unknown O serotype that showed autoagglutination (DB 02-177, isolate 03-192) and was co-infected with enteropathogenic *E. coli* (EPEC) O145:H-. Eleven (39%) of the 28 DB rabbits were positive for EPEC O145H- or O145:H7. In addition, 1 (9%) of 11 NZW rabbits from the same source was positive for *eae*-, *stx1*+ O153:H-, and O153:H7 *E. coli*. All the *E. coli* strains were negative for *stx2* and variants. In contrast, six DB rabbits from another commercial source and all four rabbits from the petting zoo were negative for EHEC and EPEC. Almost all of the *eae*-, *stx1*- *E. coli* belonged to serotype O7:H7.

### Southern Blot Analysis

The presence of the *stx1B* amplicon was confirmed in O153:H- and O153:H7 *E. coli*. The O145:H- and O145:H7 isolates were confirmed to be negative for *stx1B* (Figure 1).

### High-Resolution Genotyping by Rep-PCR

Figure 2 shows the results obtained by Rep-PCR by using two different sets of primers, REP and ERIC. All the rabbit O153:H– and O153:H7 Stx–producing *E. coli* organisms tested, including *eae*- isolates from a NZW rabbit, produced identical amplification patterns that differed from those produced by two other O153:H- EHEC strains isolated from humans. Similarly, all the rabbit O145:H- and O145:H7 EPEC isolates produced identical amplification patterns that differed from those produced by a human and a bovine O145:H- EHEC. The EHEC isolated from the rabbit infected with EPEC O145:H- had an amplification pattern similar to that produced by the O153 strains.

## Discussion

In 2001, approximately 2 million rabbits were used as a food source (9). In addition, in 2000, laboratories used ≥250,000 rabbits, and the domestic rabbit population in the United States was estimated at 9 million (9). Also, the rabbit is becoming an increasingly popular pet in U.S. households. In this study, we determined that 25% of the DB and 9% of the NZW rabbits from one commercial source of laboratory rabbits harbored Stx-producing *E. coli* O153:H-

Table. Serotypes, biochemical, and molecular characterization of *Escherichia coli* strains isolated from laboratory rabbits

| Source | Rabbit | Serotype | API code | *stx1* | *stx2* and variants | *eae* |
|---|---|---|---|---|---|---|
| A | DB 01-204 | O153:H- | 5144162 | + | - | + |
| | | O145:H- | 5144552 | - | - | + |
| A | DB 01-206 | O145:H- | 5144552 | - | - | + |
| A | DB 01-207 | O7:H- | 5144572 | - | - | - |
| A | DB 01-208 | O7:H- | 5144572 | - | - | - |
| A | DB 01-210 | O7:H- | 5144572 | - | - | - |
| A | DB 01-211 | O7:H- | 5144572 | - | - | - |
| A | DB 02-171 | O8:H- | 5144572 | - | - | - |
| | | O145:H- | 5144572 | - | - | + |
| A | DB 02-181 | O145:H- | 5144572 | - | - | + |
| | | O145:H- | 5144672 | - | - | + |
| | | O145:H- | 4144572 | - | - | + |
| A | DB 02-182 | O145:H- | 5144572 | - | - | + |
| | | O7:HM, O141:HM | 5144572 | - | - | - |
| A | DB 02-174 | O153:H- | 5144542 | + | - | + |
| A | DB 02-175 | O138:HM | 5144572 | - | - | - |
| | | O153:H- | 5144542 | + | - | + |
| A | DB 02-172 | O153:H- | 5144542 | + | - | + |
| A | DB 02-169 | O103:H2 | 5144572 | - | - | - |
| A | DB 02-177 | O145:H- | 5144572 | - | - | + |
| | | A:H- | 5144142 | + | - | + |
| | | O145:H- | 5144172 | - | - | + |
| A | DB 02-173 | O145:H- | 5144572 | - | - | + |
| A | DB 02-183 | O153:H- | 5144572 | + | - | + |
| | | O153:H- | 5144542 | + | - | + |
| A | DB 02-176 | O7:H-, O141:H- | 5144572 | - | - | - |
| A | DB 02-179 | O153:H7 | 5144542 | + | - | + |
| A | DB 02-2413 | O145:H- | 5144572 | - | - | + |
| A | DB 02-2368 | O145:H- | 5144572 | - | - | + |
| A | DB 02-10032 | O145:H- | 5144572 | - | - | + |
| A | DB 02-206 | O145:H- | 5144572 | - | - | + |
| | | O145:H7 | 5144572 | - | - | + |
| A | DB 02-207 | O145:H- | 5144572 | - | - | + |
| | | O145:H- | 5144172 | - | - | + |
| | | O145:H7 | 5144572 | - | - | + |
| A | DB 02-208 | O153:H- | 5144542 | + | - | + |
| A | DB 02-209 | O8:H44 | 5144572 | - | - | - |
| A | DB 02-210 | M:H- | 7144172 | - | - | - |
| | | O75:H- | 5144172 | - | - | - |
| A | DB 02-212 | O145:H- | 5144572 | - | - | + |
| A | NZW 02-153 | O7:H7 | 5144572 | - | - | - |
| A | NZW 02-186 | O7:H7 | 5144572 | - | - | - |
| A | NZW 02-187 | O7:H7 | 5144572 | - | - | - |
| A | NZW 02-188 | O7:H7 | 5144572 | - | - | - |
| A | NZW 02-198 | NT:H7 | 5144572 | - | - | - |
| A | NZW 02-199 | O7:H7 | 5144572 | - | - | - |
| A | NZW 02-218 | O86:H7 | 5144572 | - | - | - |
| A | NZW 02-222 | O7:H7 | 7144573 | - | - | - |
| A | NZW 02-223 | M:H7 | 7144572 | - | - | - |
| A | NZW 02-225 | O7:H7 | 5144572 | - | - | - |
| | | O145:H- | 5144572 | - | - | - |
| A | NZW 02-227 | O153:H- | 5144542 | + | - | - |
| | | O153:H7 | 5144542 | + | - | - |
| B | DB 02-228 | O75:H- | 5144172 | - | - | - |
| B | DB 02-231 | O7:H7 | 5144572 | - | - | - |
| B | DB 02-232 | O7:H7 | 5144572 | - | - | - |
| B | DB 02-233 | O7:H7 | 5144572 | - | - | - |

[a]DB, Dutch Belted; NZW, New Zealand White; A, autoagglutination; M, multiple; NT, did not react with antisera.

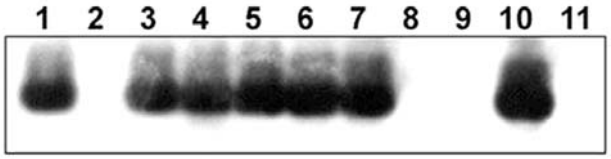


Figure 1. Southern blot analysis of DNA from rabbit *Escherichia coli* isolates by using a Shiga toxin 1B (*stx1B*) probe from rabbit enterohemorrhagic *E. coli* (EHEC) O153:H-. Lane 1, *E. coli* O157:H7 DNA (EDL 933, human isolate, positive control); lane 2, No DNA; lanes 3–5, O153:H- DNA (rabbit isolates 01-3014, 02-3283, 02-3300, respectively); lanes 6 and 7, O153:H7 DNA (02-3446 and 02-3301); lanes 8 and 9, O145:H- DNA (02-3282 and 02-3055); lane 10, rabbit isolate of unknown O serotype (03-192); lane 11, O145:H7 DNA (02-3448). *E. coli* 02-3300 and 02-3301 were *eae*- and were isolated from a New Zealand White rabbit. *E. coli* 02-3055 and 03-192 were both isolated from Dutch Belted rabbit 02-177.

or O153:H7 in their feces. These findings raise concerns about the zoonotic risk for humans, given that rabbits are common companion animals, are used for biomedical research, and are an agricultural food source. DB rabbits are very popular in pet stores, are commonly used in rabbit shows, and are the second most common rabbit breed used in research after NZW rabbits (9). In addition, NZW rabbits are commonly used as a meat source in the United States. More studies are needed to assess the prevalence of these strains and other EHEC in agricultural, pet, and wild rabbit populations. Wild rabbits were also identified as vectors of a Stx-producing O157 *E. coli* strain that was isolated from cattle in an outbreak of hemorrhagic diarrhea and HUS involving visitors to a zoo (10). Our finding that most O153:H- and O153:H7 EHEC were unable to ferment rhamnose and sucrose indicates that these biochemical markers may be useful for the detection of these strains. For example, the inability to ferment rhamnose by some human EHEC strains has been applied to the development of selective isolation media (11). Rhamnose-negative rabbit EPEC strains appear to be highly pathogenic (12).

Cattle are considered the primary reservoir host of O157 and non-O157 EHEC (13). Human infections have been linked to the presence of these bacteria in undercooked ground beef. In a recent study, the prevalence of non-O157 EHEC strains on beef carcasses was ≥50%. Some isolates in that study also belonged to serotypes O153 and O145 (13). In addition, a study investigating the prevalence of non-O157 EHEC from human diarrheal samples in the United States demonstrated that non-O157 serotypes are at least as prevalent as serotype O157 (14). Serotype O145 has been isolated from diarrheic children in the United States and is a common non-O157 EHEC serotype isolated from HUS case-patients in Europe (15,16). Indeed, non-O157 serotypes are a leading cause of HUS in Germany (17).

Co-infection of EHEC and EPEC was also identified in one rabbit in this study. The EHEC and EPEC strains isolated from this rabbit appeared to belong to different serotypes. The high prevalence of these *E. coli* strains in rabbits and the occurrence of co-infection suggest that in vivo transduction of EPEC by Stx-encoding bacteriophages from EHEC may naturally occur in this host (18). By performing Southern blot analysis, we confirmed the presence of *stx1B* in the O153:H- and O153:H7 isolates,

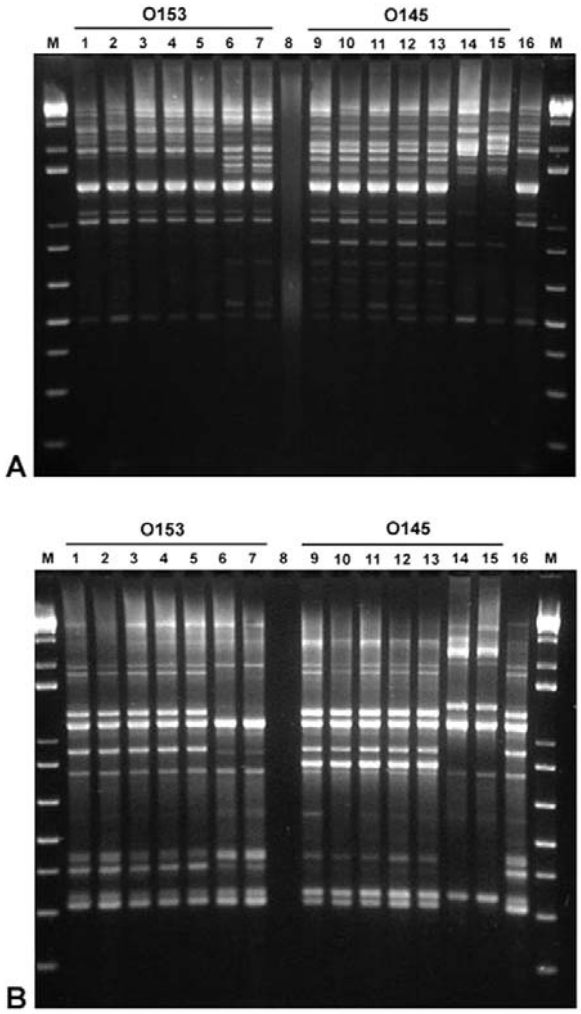


Figure 2. Repetitive-element sequence–based polymerase chain reaction analysis of genomic DNA from various *Escherichia coli* strains using REP (A) and ERIC (B) primers. Lanes 1 and 2, O153:H7 DNA (rabbit isolates 02-3446 and 02-3301, respectively); Lanes 3–5, O153:H- DNA (rabbit isolates 01-3014, 02-3050, and 02-3300); Lanes 6 and 7, O153:H- DNA (human isolates, ECRC 99-1808 and 99-1818); Lane 8, no DNA; Lanes 9 and 10, O145:H7 DNA (rabbit isolates 02-3448 and 02-3205); Lanes 11–13, O145:H- DNA (rabbit isolates 02-3750, 02-3282, and 02-3055); Lane 14, O145:H- DNA (human isolate, ECRC 95.1167); Lane 15, O145:H- DNA (bovine isolate, ECRC 95.0187); Lane 16, *E. coli* isolate of unknown O serotype (03-192) from DB rabbit 02-177; M, 1-kb plus ladder.

including the strain isolated from the rabbit infected with EPEC. In addition, by performing Rep-PCR analysis, we demonstrated that selected rabbit O153 EHEC and O145 EPEC isolates of the same O serotype represented two distinct clones. This apparent clonal nature of the isolates suggests transmission of *E. coli* between rabbits. Because these rabbit *E. coli* strains appeared to represent distinct clonal groups by Rep-PCR fingerprinting, their phylogenetic relatedness to other strains was subsequently investigated by using multilocus sequence typing. EHEC O153 and EPEC O145 belong to the EHEC 2 and EPEC 2 groups, respectively (T. S. Whittam, unpub. data). Rabbits may have become infected with these EHEC and EPEC strains from hay contaminated with cattle feces. Alternatively, rabbit-adapted *E. coli* strains may have been transduced by Stx-encoding bacteriophages from EHEC strains that transiently infected this rabbit colony.

Previous experiments in NZW rabbits, in which purified Stx1 was infused intravenously, postulated that renal lesions did not develop in rabbits and that the rabbit model failed to replicate human HUS (19,20). We recently reported that glomerulonephritis, tubular lesions, and renal glomerular thrombotic microangiopathy, the hallmark of HUS, developed in DB rabbits naturally infected with EHEC (6). In the present study, however, HUS did not develop in most rabbits colonized with EHEC. This result is consistent with human studies in which asymptomatic infection has been reported in household contacts of children with HUS and with studies showing that previous infection and frequent reexposure to *E. coli* O157:H7 may confer some protection against symptomatic illness (4,21).

In summary, our findings indicate that rabbits are a newly recognized reservoir host of EHEC that poses a zoonotic risk to humans. These findings also provide an opportunity to develop a rabbit model to study the pathogenesis of EHEC-induced disease and HUS in a naturally susceptible reservoir host.

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Dr. García is a postdoctoral fellow in the Division of Comparative Medicine at the Massachusetts Institute of Technology (MIT). His research interests include microbial pathogenesis and animal models of infection and neoplasia.

Dr. Fox is professor and director of the Division of Comparative Medicine and professor in the Biological Engineering Division at MIT. Dr. Fox's research interests are centered on microbial pathogenesis and the role of chronic inflammation in cancer.

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Address for correspondence: Alexis García, Division of Comparative Medicine, Massachusetts Institute of Technology, 77 Massachusetts Avenue, 16-820, Cambridge, MA 02139, USA; phone 617-253-1757, fax: 617-258-5708; email: agarcia@mit.edu



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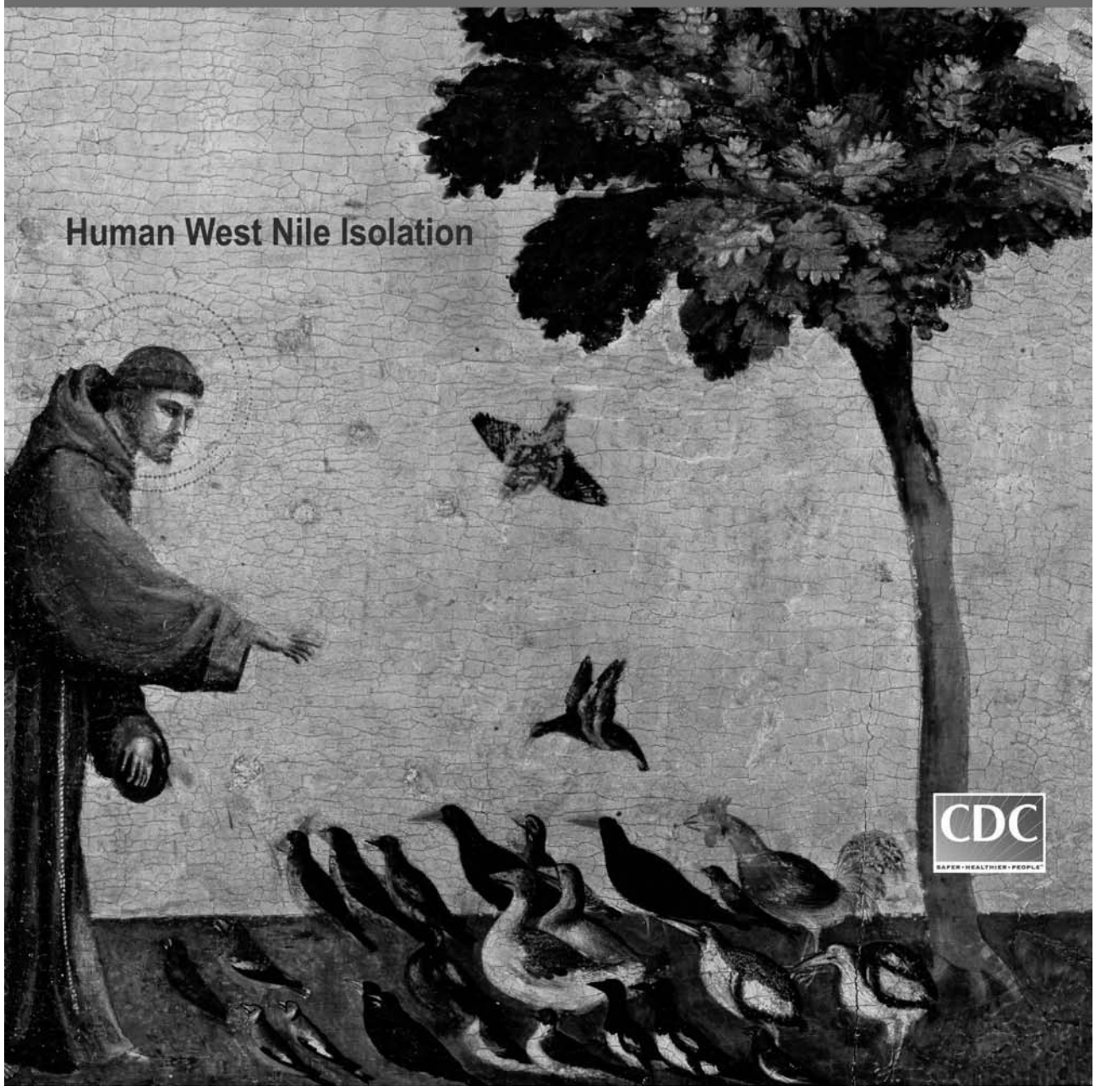

# Alexander the Great and West Nile Virus Encephalitis

**John S. Marr* and Charles H. Calisher†**

Alexander the Great died in Babylon in 323 BC. His death at age 32 followed a 2-week febrile illness. Speculated causes of death have included poisoning, assassination, and a number of infectious diseases. One incident, mentioned by Plutarch but not considered by previous investigators, may shed light on the cause of Alexander's death. The incident, which occurred as he entered Babylon, involved a flock of ravens exhibiting unusual behavior and subsequently dying at his feet. The inexplicable behavior of ravens is reminiscent of avian illness and death weeks before the first human cases of West Nile virus infection were identified in the United States. We posit that Alexander may have died of West Nile virus encephalitis.

Alexanderthe Great died in the ancient Mesopotamian city of Babylon, on June 10, of 323 BC (Figure). His death after a 2-week febrile illness (Table) has fascinated ancient scholars and contemporary medical investigators (1), who have posited various diagnoses based on sparse clinical information—a few recorded signs and symptoms. Retrodiagnoses have included poisoning and infectious as well as noninfectious diseases (1–6). After reviewing ancient accounts and modern theories, we have concluded that Alexander may have died of West Nile virus encephalitis .

## Previous Theories

### Poisons

Few poisons induce fever, and few of these were available in Alexander's time—except plant salicylates, which disturb temperature regulation; alkaloids, which interfere with perspiration; and ergot mycotoxins, which produce a subjective sensation of heat**.** Plutarch mentions that Aristotle (Alexander's tutor) procured arsenic to poison Alexander (7). But plants, mycotoxins, and arsenic are not the likely causes of death since none would have caused the reported high, sustained fever.

*Virginia Department of Health, Richmond, Virginia, USA; and Colorado State University, Fort Collins, Colorado, USA

### Infections

Alexander's death occurred in late spring, upon his return to Babylon from the Indian subcontinent. Environmental conditions were unremarkable (8). Babylon, located on the Euphrates River (90 km south of present-day Baghdad), was bordered on the east by a swamp. Animals, including birds, were abundant (9), and arthropods were also likely present (available from: URL: http://www.ac.wwu.edu/~stephan/Animation/alexander.html). Diseases endemic to the area (present-day Iraq) (leishmaniasis, bubonic plague, hemorrhagic fevers) were not mentioned by chroniclers of Alexander's death. Also not reported was illness among his troops, mainly Macedonians and local recruits. Descriptions of Alexander's illness do not include common disease signs (e.g., rash, icterus, "thin blood," vomiting, diarrhea or dysentery, hematuria, seizures).

Malaria, a diagnosis postulated by previous authors (1–3), occurred in Mesopotamia (10,11), and is common in today's Middle East (12). Some of Alexander's symptoms


Figure. Map of Mesopotamia (present-day Iraq), including its capital, Babylon.

Table. Medical history and physical examination of Alexander the Great

| Patient characteristics | Medical history | Clinical symptoms |
|---|---|---|
| Male | Ten years before death, traveled widely (Mediterranean, North Africa, and Middle East) | Escalating fever associated with chills |
| Born in Macedonia | Unexplained fever 5 years previously | Excessive thirst, diaphoresis |
| 32 years of age | Penetrating right chest wound one year before final illness | Acute abdominal pain |
| Soldier | Onset of final illness May 29, 323 BC | Single episode of back pain at onset of fever |
| Heavy drinking | Death June 10, 323 BC | Increased weakness leading to prostration with intermittent periods of energy |
| Frequent bathing | | Delirium |
| Married to many wives | | Aphonia |
| One son | | Terminal flaccid paralysis |

are compatible with malaria: continuous fever, chills, diaphoresis, prostration, myalgia, progressive weakness, stupor, diminished sensorium, delirium; however, dark urine, so called "black water fever," or intermittent fevers were not reported. Today, most malaria in Iraq is due to *Plasmodium vivax* (13). Given Alexander's travel history, had his illness been malaria, it would have been due to *P. falciparum*; however, absence of *P. falciparum*'s dramatic signature fever curve diminishes the possibility of malaria as a probable cause.

Typhoid fever and its complications also have been thoroughly considered (1). Alexander had a 2-week febrile illness culminating in terminal encephalopathy. As do encephalitis, endocarditis, pneumococcal pneumonia, psittacosis, rickettsial disease, and tularemia, typhoid causes sustained or continuous fever (14). The typical course of typhoid fever lasts one month. In fatal cases, death usually occurs at the end of week 2. Typhoid's neurologic manifestations, which also include delirium and expressionless demeanor, are seen in week 3. Other signs include cough, diarrhea, "rose spots," epistaxis, and bloody stool (15). None of these signs or other illnesses similar to Alexander's were documented by Plutarch. Most other enteric infections have no neurologic sequelae and are generally self-limited. *Vibrio vulnificus* infection, which may cause fatal sepsis in heavy drinkers (as was Alexander), causes rapid death, accompanied by skin and muscle lesions and bleeding.

Other suggested diagnoses include *Schistosoma haematobium* infection (4), which causes painless hematuria; however, ectopic egg deposition may occur at any time, causing transverse myelitis, paralysis, and death (16). Exposure to cercariae produces pruritus and Katayama fever induces serum sickness (4), but symptoms include low grade fever and pruritic swellings, which were not reported in Alexander's case. Some leptospirosis symptoms are consistent with Alexander's illness; however, other classic leptospirosis signs (biphasic fever, calf or thigh pain, jaundice, hemorrhage, pulmonic involvement) were not reported. *Acanthamoeba* spp. (pathogenic free-living amoebae) and *Naegleria* spp. cause meningoencephalitis, which is acquired during bathing, an activity in which Alexander reportedly participated with compulsion. Acanthamoebae are cosmopolitan but prefer compromised hosts. Moreover, death from naegleriasis usually occurs within a week of onset, and encephalitis caused by acanthamoebae causes death only after a prolonged period of symptoms.

When Alexander's clinical symptoms were listed on GIDEON (Global Infectious Diseases and EpidemiOlogy Network (13), influenza ranked highest (41.2% probability) on the list of differential diagnoses. While influenza could have killed Alexander, reports did not mention others becoming ill with similar symptoms. Lymphocytic choriomeningitis, an influenzalike illness followed by meningoencephalitis, is rare. Poliomyelitis can occur as an isolated case or as an epidemic; its characteristics include fever, vomiting, severe myalgia, and prostration, as well as the early complication of flaccid paralysis, which has been postulated as another late sign in Alexander's illness (1). This interpretation narrows the differential diagnosis to include poliomyelitis (see above), Guillain-Barré syndrome, and the encephalitides. (A list of the many other infectious diseases others have considered as well as additional, less likely candidates is available from the authors.)

## West Nile Fever and Encephalitis

West Nile fever was not considered by previous authors as cause of Alexander's death, possibly because it has only recently emerged globally. West Nile virus (family *Flaviviridae*, genus *Flavivirus*), first isolated from a febrile patient in Uganda in 1937 (17), is one of many viruses causing encephalitis. Infection is marked by fever, encephalitis, or meningoencephalitis. Until the early 1990s,

the virus was largely confined to Africa, Europe, and Asia. In 1941, an outbreak occurred in Tel Aviv, with no deaths reported. Over the next 60 years, seven outbreaks occurred in Israel and its environs (18). In 1957, during an outbreak in an army camp, a single case of encephalitis was recognized in a group of 300 soldiers (19). By 2000, a countrywide outbreak occurred, with a case-fatality rate of 8.4% (20). In 1999, West Nile virus was introduced to the United States, and 4,156 laboratory-confirmed human cases of infection (earliest onset of illness, June 10) occurred in 2002 (21). Median age in fatal cases was 72 years, although neurologic disease occurred in persons of all ages. Also recognized in both fatal and nonfatal cases was flaccid paralysis in patients with encephalitis.

West Nile virus infections in vertebrates may have been occurring in the Middle East for centuries. Now the virus has spread to new areas of the world and to new populations and causes infection characterized by new signs and symptoms. In the 2000 epidemic in Israel, encephalitis occurred in nearly 59% of 417 human cases. Of 233 hospitalized patients (case-fatality rate 14%), >98% had fever, 46% cognitive changes, and 17% abdominal pain or myalgias. Nearly 18% became comatose (22). Acute flaccid paralysis was noted, as in the United States in 1999 and later (23).

When West Nile virus–infected *Culex* spp. mosquitoes take a blood meal from a susceptible vertebrate, the virus may be incidentally transmitted. Birds serve as amplifying hosts, the degree of amplification depending on avian species, environmental conditions, and other factors. Birds with viremia provide mosquitoes blood meals; these mosquitoes subsequently serve to bridge West Nile virus infection to humans. Responses to recent epizootics and epidemics have improved our understanding of the disease. New, competent mosquito vectors are recognized, new human and mammalian symptoms are identified, and new bird species are determined as poor, intermediate, or excellent amplifiers of the virus.

Ludwig et al. examined 437 birds at the Bronx Zoo and Wildlife Conservation Park during the 1999 West Nile virus epizootic and epidemic in New York City (24), where virus activity was first recognized in wild and captive birds in the United States. Avian deaths were observed weeks before the first human West Nile virus encephalitis cases. Even though 42% of birds tested were New World birds, 14 (82%) of 17 deaths were in New World birds and 3 (5%) of 57 were in Old World birds, which suggests that birds in the latter group might have had innate immunity by virtue of their ancestral, coevolutionary history with the virus. Diseased birds manifested various symptoms, including abnormal head and neck posture, ataxia, tremors, circling, disorientation, and impaired vision. Most birds with symptoms died.

In Iraq, several mosquito species, including *Culex tritaeniorhynchus*, *Cx. theileri*, and *Aedes caspius* (25) have been implicated in West Nile virus transmission. Although mosquitoes in Iraq have not been completely catalogued, it is likely that, as in the United States, other mosquitoes there also serve as vectors of West Nile virus. Mosquitoes are found throughout Iraq, from March to December, and have various larval habitats. Annual spring flooding of the Tigris and Euphrates rivers provides ideal breeding grounds for *Culex* spp. Mosquito species that may have occurred in Babylon are unknown; however, breeding habits must be ancient, and mosquitoes are well known for their proclivity to breed in swamps.

Still, the possibility that West Nile virus killed Alexander is mitigated by the fact that he fell ill in May. Although the virus may have occurred there at that time, most recent human cases in Israel occurred in July to September, with only a few cases occurring in June. In temperate areas, West Nile virus infection in humans is seasonal. Amplification occurs in mosquitoes and birds several months before the virus spills over into dead-end hosts. Experimentally infected indigenous mosquitoes showed an intrinsic incubation period of 7 to 14 days at 28°C (26). Others have shown that when *Cx. pipiens* mosquitoes were allowed to feed on viremic chicks infected with West Nile virus and incubated at 30ºC virus could be detected 4 days later (27). This suggests that maximum virus amplification may not be reached until mid-summer. Iraq's mean high spring temperature is 29ºC (28), somewhat higher than Tel Aviv's (24ºC).

Israel has had West Nile virus activity and human cases during the last 3 years, with most human cases not detected until August. Israel is at the same latitude as Iraq and has similar climate. If Iraq also had slightly higher temperatures 2,000 years ago (we will never know this with certainty), onset of disease in humans and birds, including inexplicable avian die-offs, could have occurred earlier in the summer. We reread Plutarch and saw the following passage about Alexander's entrance into Babylon: "… when he arrived before the walls of the city he saw a large number of ravens flying about and pecking one another, and some of them fell dead in front of him." (29)

Bird observers (*dagil issuri*) were common in Asia Minor at the time. These diviners considered birds as oracles. Greek *Kulturkreis* and Babylonian *Alalakh* tablets mention auguries based on the behavior of birds, particularly fighting birds, to predict the future (30). Plutarch presumably thought it sufficiently noteworthy to record angry or disoriented ravens, although it is impossible to determine whether this event was added later as a necessary metaphoric foreboding of Alexander's death.

Current geographic distribution of corvids indicates that these likely were ravens (*Corvus corax*) and not crows

(*Corvus corone sardonius* or other crow species). No ravens were at the Bronx Zoo in 1999 (T. MacNamara, Wildlife Conservation Society, and pers. comm., 2003). However, in the United States today, New World crows (American crow, *C. brachyrhynchos* and fish crow, *C. ossifragus*) are among the birds most susceptible to fatal West Nile virus infections. One wonders if an influx of migratory birds might have served as reservoirs of West Nile virus and directly or indirectly (through mosquitoes) infected ravens in Babylon, causing a massive die-off.

Pathogenicity of West Nile virus for corvids was established 50 years ago. Work et al., assigned by the Rockefeller Foundation to study arboviruses in Egypt, isolated 23 West Nile virus strains from blood samples of febrile children in the Sindbis area and found that the virus caused illness in more children and young adults than in older adults. In addition, and particularly germane to our hypothesis, they isolated West Nile virus from a hooded crow (*Corvus corone sardonius*) for the first time, demonstrating experimental infection of birds with viremia as high as $10^9$, and death rates of 100% (31,32). During winter, 80% of these crows were seropositive, and the investigators assumed that during transmission season, crow death rates were high. The experimental studies showed that mosquitoes could be infected by feeding on hooded crows with viremia levels as low as $10^{3.5}$ and could subsequently serve as West Nile virus vectors to humans of any age. This early epidemiologic work provided an early clue in New York City in 1999, when both exotic and domestic birds signaled the introduction of West Nile virus disease to the New World (33). Before 1998, the virus was not recognized as an important cause of death in wild birds; therefore, it was surprising to find that the Israeli 1998 strain was the same as that which infected birds at the Bronx Zoo. Ravens dropping dead from the skies likely were also a surprise to Alexander.

## Conclusions

Alexander the Great died in late spring in the semi-tropical, urban area of present-day Baghdad. Explanations for his death have included poisoning, enteric and parasitic diseases, influenza, and poliomyelitis. Our diagnosis, as well as previous alternative diagnoses, may be subject to author bias, errors in translation, and a paucity of clinical information. We assumed that he died in late spring in Babylon after a 2-week illness that included fever and signs suggestive of encephalitis. We presumed that diseases now endemic to Iraq were also present in ancient Mesopotamia. Recent scholarly thought has been ingenious and rigorous, given the sparseness of available information. Nonetheless, earlier diagnoses did not include West Nile virus encephalitis. Previous considerations omitted an event that was carefully recorded by Plutarch and which, before 1999, might have been considered irrelevant: the erratic behavior and observable deaths of numerous ravens outside the walls of Babylon. This observation might now be construed as an important clue. If this observation is included as part of the description of Alexander's illness, West Nile virus encephalitis complicated by flaccid paralysis becomes an alternative diagnosis. It is possible that, in the 3rd century BC, disease caused by West Nile virus arrived in Mesopotamia for the first time in recorded history, killing indigenous birds and an occasional human and causing only incidental febrile illnesses in many others. Over subsequent centuries the virus may have devolved, becoming less pathogenic for indigenous birds, while retaining its potential as a dangerous human pathogen. This is speculative, but in 1999, a "natural experiment" did occur when an Old World epizootic strain was introduced into the United States. What has been observed in the ongoing North American epizootic and epidemic might be similar to what happened in Babylon many years ago. We now know that unexplained bird die-offs can presage human cases of disease caused by West Nile virus. In 323 BC, a similar event might have been considered an omen of Alexander the Great's death. In this instance, the oracles would have been correct.

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Dr. Marr is director of the Office of Epidemiology in the Virginia Department of Health. His research interests include medical history. He has written articles on possible causes of the 10 plagues of Egypt, the Mexican *huey cocolitzlti* epidemic of 1596, and the mysterious epidemic preceding the death of the last Incan emperor, Hayna Capac.

Dr. Calisher is professor of microbiology at the Arthropod-borne and Infectious Diseases Laboratory, Colorado State University. His research interests include disease epidemiology, virus evolution, everything about arborviruses, and the epidemiology of rodent-borne viruses.

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Address for correspondence: Charles H. Calisher, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA; fax: 970-491-8323; email: calisher@cybercell.net

# West Nile Virus in Mexico: Evidence of Widespread Circulation since July 2002

**José G. Estrada-Franco,***
**Roberto Navarro-Lopez,† David W.C. Beasley,***
**Lark Coffey,* Anne-Sophie Carrara,***
**Amelia Travassos da Rosa,* Tamara Clements,‡**
**Eryu Wang,* George V. Ludwig,‡**
**Arturo Campomanes Cortes,†**
**Pedro Paz Ramirez,† Robert B. Tesh,***
**Alan D.T. Barrett,* and Scott C. Weaver***

West Nile virus (WNV) antibodies were detected in horses from five Mexican states, and WNV was isolated from a Common Raven in the state of Tabasco. Phylogenetic studies indicate that this isolate, the first from Mexico, is related to strains from the central United States but has a relatively high degree of sequence divergence.

During the summer of 2002, the Agricultural Ministry of Mexico (SAGARPA) received reports of encephalitis-like illness in horses from several different areas of Mexico, concurrent with reports of West Nile virus (WNV) encephalitis outbreaks in horses along the Texas border in the states of Coahuila, Tamaulipas, and Chihuahua. Other suspected cases were reported from several southern, tropical states. We report the results of an equine serosurvey conducted from July 2002 to March 2003 by the Office of Exotic Diseases of the Agricultural Ministry (CPA-SAGARPA). We also describe the first isolation of WNV in Mexico, in a Common Raven (*Corvus corax*) from the state of Tabasco.

The Republic of Mexico is divided by the Tropic of Cancer, with temperate, arid climate zones in the north and at higher elevations and humid, subtropical, and tropical climate zones in the south. Our study encompassed most of these climatic zones, as equine serum samples were collected from 3 border states, 1 state on the Tropic of Cancer, and 10 states south of the Tropic of Cancer (Figure 1). Sampled equine populations were chosen on the basis of a history of clinical encephalitis; medical history was provided by owners and corroborated by CPA-SAGARPA veterinarians. In total, 441 serum samples were analyzed for WNV antibodies.

Because most serum samples were collected late in the probable virus transmission season, all were first screened for immunoglobulin (Ig) G antibodies, using IgG enzyme-linked immunosorbent assays (ELISA) with a recombinant, envelope protein domain III antigen expressed and purified from *Escherichia coli* (D.W.C. Beasley, et al., submitted for pub.). Positive samples were confirmed by hemagglutination inhibition (HI) tests against WNV and St. Louis encephalitis virus (SLEV), by 90% plaque reduction neutralization tests (PRNT) against WNV (1), and by ELISA with WNV, SLEV, and Venezuelan equine encephalitis virus (VEEV) antigens and viruses. (The presence of several endemic arboviruses, including SLEV and VEEV, necessitated additional testing.) WNV infection was confirmed if the WNV antibody titer was ≥fourfold higher than the SLEV titer. To investigate evidence of recent WNV infection, 198 samples were also tested by using both IgG- and IgM-specific ELISA with WNV-infected cell culture antigens (2). Selected samples were tested by ELISA and PRNT for VEEV antibodies to determine if this virus was circulating in areas reporting equine encephalitis.

## Results

A total of 441 equine serum samples from 14 states of Mexico were tested (Figure 1). WNV-specific antibodies were detected in 97 (22%) of the samples. These data probably overestimate the true equine seropositivity rate because sampling focused on herds with a history of clinical encephalitis. Representative data from 22 of the WNV-positive samples obtained in five different states are pre-

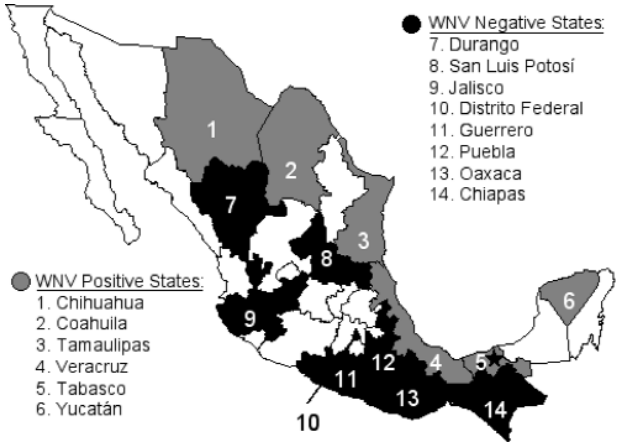


Figure 1. Map showing the Mexican states sampled for antibodies to West Nile virus and Venezuelan equine encephalitis virus in equines. Unshaded states were not sampled. The location of the West Nile virus isolation from a dead Common Raven is shown by a star.

*University of Texas Medical Branch, Galveston, Texas, USA; †Comision Mexico-Estados Unidos para la Prevencion de la Fiebre Aftosa y Otras Enfermedades Exoticas de los Animales, Mexico City, Mexico; and ‡U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA

sented in Table 1. No evidence was obtained of SLEV infection in equines, but VEEV-specific antibodies were detected in serum samples from the states of Veracruz and Yucatán. (Horses are vaccinated against VEEV in the states of Chiapas and Oaxaca, so samples from these locations were not tested for VEEV antibodies.) The positive samples, several of which also contained WNV IgG, represent natural VEEV circulation and infection of horses, presumably with enzootic subtype IE strains (3,4).

On May 5, 2003, the CPA-SAGARPA received a report of a dead Common Raven from the El Yumka wildlife preserve in the city of Villahermosa, state of Tabasco. Although this species is native to Tabasco and other regions of Mexico, this bird was one of two ravens imported from the United States in 1999. A necropsy was performed, and virus isolation was attempted on tissue samples at the CPA-SAGARPA biosafety level 3 facility in Palo Alto, Mexico City. On May 16, 2003, cytopathic effects were detected in Vero cells injected with brain suspension. Viral RNA from the isolate was genetically characterized at the National Institute for Epidemiology and Diagnostics (InDre) in Mexico City and at the University of Texas Medical Branch in Galveston. A 2,004-nt genome portion, including the prM-E protein region, was amplified by using a reverse transcription–polymerase chain reaction as described previously (5); the resulting amplicons were sequenced directly with the Big Dye sequencing kit and model 3100 sequencer (Applied Biosystems, Foster City, CA). The sequence of this WNV isolate (TM171-03, submitted to GenBank under accession no. AY371271) was aligned with all homologous WNV sequences of the same length available from the GenBank library (homologous to nt 466–2,469 in the Flamingo382 strain, GenBank accession no. AF196835), and phylogenetic trees were constructed by using maximum parsimony, maximum likelihood (incorporating empirical base frequencies, a general time-reversible substitution model with the following frequencies: A→C 1.34263; A→G 4.18575; A→U 1.55497; C→G 0.044980; C→T 15.08737; G→U 1.00000, a g shape parameter of 0.228), and neighbor joining programs implemented in the PAUP 4.0 software package (6). All trees had nearly identical branching orders; the maximum parsimony tree is presented in Figure 2. All trees placed the Mexican raven isolate as a sister to a clade that comprises most WNV strains isolated in Texas during 2002. Two other WNV strains from the Bolivar Peninsula, Texas (362, 476), were positioned basally to a 1998 Israeli stork isolate that grouped with all other North American isolates, suggesting that the North American strains may not all have originated from a point source introduction

Table 1. Serologic analysis[a] of 22 horse serum samples from five Mexican states, positive for West Nile virus antibodies, July 2002–March 2003[b]

| | | | | | WNV serologic findings | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| State, locality | Sample no. | Date | Age | Sex | IgG-E | IgM | IgG | PRNT | HI | HI SLEV |
| Veracruz, Minatitlan | VER-015 | Oct 28, 2002 | 7 y | M | pos | 1,600 | 400 | >320 | 20 | 20 |
| Veracruz, Minatitlan | VER-011 | Oct 28, 2002 | 18 m | M | pos | neg | 400 | >320 | 160 | neg |
| Veracruz, Hidalgotitlan | VER-017 | Oct 29, 2002 | 8 m | M | pos | 12,800 | neg | >320 | >640 | 160 |
| Veracruz, Texistepec | VER-26 | Oct 29, 2002 | 2 y | M | pos | neg | 100 | 40 | 160 | neg |
| Veracruz, Texistepec | VER-025 | Nov 7, 2002 | 18 m | M | pos | 6,400 | neg | >320 | >640 | 160 |
| Veracruz, J. Carranza | VER-036 | Nov 11, 2002 | 7 m | M | pos | neg | 6,400 | >320 | >640 | 40 |
| Veracruz, Angel R. Cabada | 406 | Mar 20, 2003 | 13 m | F | pos | neg | 400 | >320 | 80 | neg |
| Veracruz, Cosamaloapan | 187 | Aug 28, 2002 | 5 y | F | pos | neg | 1,600 | >320 | 160 | 20 |
| Yucatan, Merida | 389 | Mar 7, 2003 | 4 y | F | pos | neg | 1,600 | nt | 80 | 20 |
| Yucatan, Tizimin | 391 | Mar 1, 2003 | 5 y | M | pos | neg | 1,600 | 160 | 40 | 20 |
| Chihuahua, Ojinaga | 210 | Oct 20, 2002 | 2 y | M | neg | 1,600 | neg | 40 | 320 | 40 |
| Coahuila, Cd Acuna | 112 | Oct 14, 2002 | 3 y | M | pos | 1,600 | neg | 80 | 20 | 20 |
| Coahuila, Hidalgo | 4 | Sep 1, 2002 | 5 | F | pos | 400 | 100 | >320 | >640 | 20 |
| Coahuila, Villa Union | 26 | Nov 2, 2002 | 4 y | M | pos | 400 | 100 | >320 | 320 | 80 |
| Coahuila, Nava | 67 | Jul 11, 2002 | 7 y | M | pos | 400 | 100 | >320 | 320 | 20 |
| Coahuila, P. Negras | 56 | Nov 5, 2002 | 2 y | M | pos | 6,400 | 100 | >320 | >640 | 40 |
| Coahuila, Zaragoza | 39 | Oct 10, 2002 | 5 y | F | pos | neg | 400 | >160 | 320 | 20 |
| Coahuila, Morelos | 51 | Oct 5, 2002 | 7 y | M | pos | neg | 100 | >160 | 160 | 40 |
| Tamaulipas, Diaz Ordaz | 268 | Nov 7, 2002 | 8 y | F | pos | 12,800 | 1,600 | 40 | 320 | 40 |
| Tamaulipas, Camargo | 279 | Nov 11, 2002 | 4 y | F | pos | 12,800 | 3,200 | >320 | 320 | 80 |
| Tamaulipas, Rio Bravo | 349 | Nov 5, 2002 | 8 y | M | pos | neg | 400 | 80 | 80 | neg |
| Tamaulipas, Victoria | 344 | Nov 7, 2002 | 2 y | M | pos | neg | 6,400 | >320 | 160 | 20 |

[a]Titers expressed as reciprocal of dilution; all tests were enzyme-linked immunosorbent assays unless otherwise noted; all tests were against WNV (West Nile virus) unless otherwise noted.
[b]SLEV, St. Louis encephalitis virus; Ig, immunoglobin; G-E, recombinant domain III of E protein; PRNT, plaque reduction neutralization test; HI, hemagglutination inhibition test.

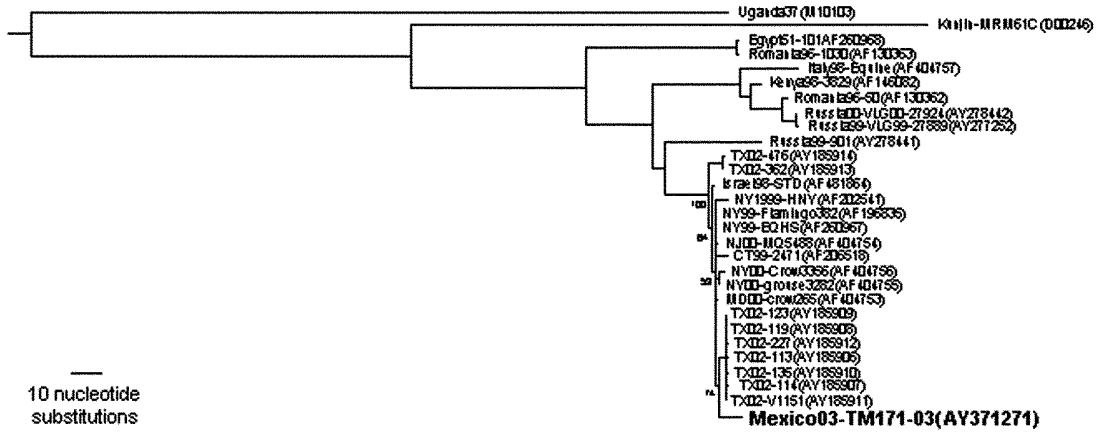


Figure 2. Phylogenetic tree, derived by maximum likelihood (ML) by using prM-E sequences for West Nile virus (WNV) isolates including the 2003 Mexican Raven isolate. Strains are indicated by country or abbreviated state (U.S.) followed by year and strain designation. GenBank accession nos. are in parentheses. A lineage 2 WNV strain (AY277251) was used to root the tree. Numbers indicate bootstrap values from 1,000 replicates.

into the New York area in 1999. This topologic finding was the result of a single synonymous, third codon position (genomic position 969 by using the numbering of the Flamingo382 isolate, GenBank accession no. AF196835) U nucleotide (synapomorphy) shared by the 1998 Israeli and all North American strains except the Bolivar Peninsula isolates. However, the relatively poor support, represented by bootstrap values (1,000 replicates) of only 56–59 by using the three different phylogenetic methods, suggests that this topologic finding is not necessarily correct. More robust phylogenetic analyses using complete genomic sequences are needed to clarify these relationships.

Comparison of nucleotide sequences indicated mutations at 9 nt (0.45%) of the Mexican TM171-03 sequence compared to the prototype NY99 strain (Flamingo382, GenBank accession no. AF196835). Some of these nucleotide positions vary among other strains sampled worldwide, suggesting that they are not under strong purifying selection. Comparison with sequences of Year 2002 Texas strains showed only one shared mutation (genomic position 2466 C→U). WNV has remained genetically conserved in the New World; however, based on our limited sequencing, the Mexican strain appears to be the most divergent WNV isolate identified to date in the Americas (Table 2). Two of the mutations resulted in amino acid changes at prM141 (Ile→Thr) and E156 (Ser→Pro), which have not been reported to date in North American isolates. The Ser→Pro amino acid substitution at residue E156 is of interest, as it abolishes a potential glycosylation site that is a putative WNV virulence determinant (7–9). This report is the first of a New World WNV isolate with probable altered E protein glycosylation. Further studies are in progress to assess the possible phenotypic effects of this mutation.

## Conclusions

Two recent publications reported serologic evidence of WNV infection among equines in the states of Yucatán and Coahuila from serum samples collected beginning in July and December 2002, respectively (10,11). We obtained serologic evidence of more widespread circulation of WNV in five other Mexican states, also dating back to July 2002. We also report the first isolation of WNV from Mexico from a dead Common Raven that resided in a wildlife preserve in Tabasco.

Genetic studies indicated that the Mexican WNV strain was likely introduced from the central United States. The level of genetic divergence (9 nt) of the Mexican isolate and the unique amino acid substitutions in the prM and E proteins when compared to all other North American WNV isolates suggest that the Mexican strain has been evolving independently for some time and did not simply enter Mexico recently from Texas. We speculate that this strain descended from a WNV strain introduced into the Yucatán peninsula by migrating birds. Nucleotide

Table 2. Nucleotide and deduced amino acid differences in the prM-E gene region between the prototype New York (382-99) and Mexican (TM171-03) West Nile virus strains

| Nucleotide (amino acid)[a] | Strain 382-99 | Strain TM171-03 |
|---|---|---|
| 483 | C | U |
| 858 | C | U |
| 887 (prM141) | U (Ile) | C (Thr) |
| 1137 | C | U |
| 1432 (E156) | U (Ser) | C (Pro) |
| 1626 | C | U |
| 2328 | C | U |
| 2388 | C | U |
| 2466 | C | U |

[a]Nucleotide numbering used for the New York flamingo 382-99 sequence (Genbank accession no. AF196835); locations of encoded amino acid differences are shown in parentheses.

sequences of viruses isolated from Mexican states close to the U.S. border, once obtained, may more closely resemble strains isolated in Texas during 2002.

Of particular interest is the overlap in distribution of WNV and VEEV (serologic data for VEEV not shown) in the southern Mexican states of Veracruz and Yucatán; the presence of other flaviviruses like SLEV in these states is also likely. Both WNV and VEEV produce clinically similar neurologic disease in horses, and past, presumptive diagnoses of VEEV may have been inaccurate. Steps are now in place at the Mexico City headquarters of the Animal Health Division of SAGARPA to implement appropriate laboratory diagnosis for flaviviruses and alphaviruses. Additionally, field personnel are instructed to investigate epidemiologic signs of possible WNV infection including avian death and unusual human neurologic syndromes.

The biologic and epidemiologic consequences of mosquito-borne encephalitis viruses (12) cocirculating in the same ecosystem should be examined. The impact of WNV on human health in regions (such as Mexico) where inhabitants may have extensive prior exposure to other flaviviruses such as dengue, SLEV, Ilheus, Bussuquara, Jutiapa, and Yellow fever viruses may differ from that in regions (e.g., the United States and Canada) where human exposure to flaviviruses is very limited. WNV infection in persons with previous flavivirus immunity, which could either attenuate disease because of cross-protective antibodies (13) or potentially worsen disease because of immune enhancement (14), should be studied. Our ongoing VEEV surveillance in southern Mexico may identify differences in transmission habitats for VEEV and WNV and assist with optimizing virus containment efforts.

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Dr. Estrada-Franco is an assistant professor at the University of Texas Medical Branch. His research interests include the ecology and epidemiology of vector-borne diseases, their human impact, vector genetics, and vector-host-pathogen interactions of arboviruses and parasitic diseases.

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Address for correspondence: Scott Weaver, Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-0609, USA; fax 409-747-2415; email: sweaver@utmb.edu

# Severe Acute Respiratory Syndrome Epidemic in Asia

**Guofa Zhou* and Guiyun Yan***

We analyzed the dynamics of cumulative severe acute respiratory syndrome (SARS) cases in Singapore, Hong Kong, and Beijing using the Richards model. The predicted total SARS incidence was close to the actual number of cases; the predicted cessation date was close to the lower limit of the 95% confidence interval.

As of May 15, 2003, the cumulative number of reported probable cases of severe acute respiratory syndrome (SARS) was ≥7,600 worldwide (1). In the 28 countries reporting SARS cases, the People's Republic of China (PRC), particularly the Hong Kong Special Administrative Region and the Beijing Municipality, reported most of the cases. The Beijing municipal government took various measures to prevent the spread of SARS. As in Hong Kong (2,3), measures in Beijing included wearing masks and handwashing, mandatory home quarantine of persons who had contact with probable SARS patients, suspension of schools and universities for 2 weeks, restrictions on public gatherings, screening body temperatures of air travelers, discouragement of mass migration by air or train, designation of special hospitals for the treatment of SARS patients, and education on SARS transmission and personal protection. The number of new cases reported daily in Beijing were high (e.g., 39 new cases on May 14, 2003), and public and health authorities were concerned about how extensive the SARS epidemic might be and when the SARS epidemic might be brought under control if intervention measures were continued.

## The Study

We examined the dynamics of reported SARS clinical cases in three cities in Asia (Beijing, Hong Kong, and Singapore) and used the Richards model (4) to predict SARS infection over several months. For Hong Kong and Singapore, data on SARS cases were extracted from daily reports of the World Health Organization (WHO) from March 17 to May 15, 2003 (1). For Beijing, the number of confirmed SARS cases was published by the Ministry of Health of PRC (5). (The PRC's report was used because WHO did not report SARS case incidence in Beijing; the WHO report summarized the total number of SARS cases in mainland China.) Although SARS case reporting started in early April 2003 in Beijing, the accuracy of SARS daily case reporting in Beijing before April 21 was questioned (6); thus our analysis for Beijing was based on case numbers from April 21 to May 15, 2003. Data indicated that daily new SARS cases were declining since April 12, April 2, and April 29 in Singapore, Hong Kong, and Beijing, respectively (Figure). The cumulative cases in all three localities resembled S-shaped curves (Figure).

When $S(t)$ is used to represent the cumulative number of SARS cases on day $t$, the dynamics of S can be modeled as

$$\frac{dS}{dt} = r\frac{S}{F(S)},$$

where $r$ is the intrinsic growth rate, and $F(S)$ measures the effectiveness of intervention measures. The basic reproductive number of an infection, $R_0$ (defined as the average number of secondary cases generated by one primary case), can be estimated as $R_0 = e^{(rT)}$, where $T$ is the generation time of an infection. This model assumes that 1) the rate of cumulative SARS case increase is proportional to the present number of cases, 2) without control measures the SARS case incidence grows exponentially, and 3) intervention measures will have a negative effect on SARS case increase. This model does not take into account spatial and stochastic processes of SARS transmission. $F(S)$ can be expressed as

$$\frac{1}{F(S)} = 1 - \left(\frac{S}{K}\right)^{a},$$

where $K$ is the maximum cumulative case incidence, and $\alpha$ measures the extent of deviation of S-shaped dynamics from the classic logistic growth model ($\alpha = 1$). $\alpha > 1$ or $\alpha < 1$ indicates that the cumulative case numbers grow faster or slower than predicted by the logistic growth model (4). The explicit solution of the model is

$$S = \frac{K}{\left(1 + e^{-r(t - t_m)}\right)^{1/\alpha}}, \text{ where } t_m = \frac{1}{r} Ln\left(\left[\frac{K}{S_0}\right]^{a} - 1\right),$$

and $S_0$ is the number of cases at $t = 0$. Parameter $t_m$ is the inflection point where maximum growth rate occurs; in the case of logistic growth model S = K/2 when t = $t_m$. This model predicts that the cumulative SARS case incidence follows an S-shaped curve and gradually reaches a maximum case incidence, $K$. The end of the epidemic is defined as not a single new SARS case emerging in 3 consecutive months (7). The earliest time to reach this point, $t_0$, is calculated through the numerical solution of inequality

$$\int_{t_0}^{t_0+90} (K - S(t))dt < 1.$$

*State University of New York at Buffalo, Buffalo, New York, USA

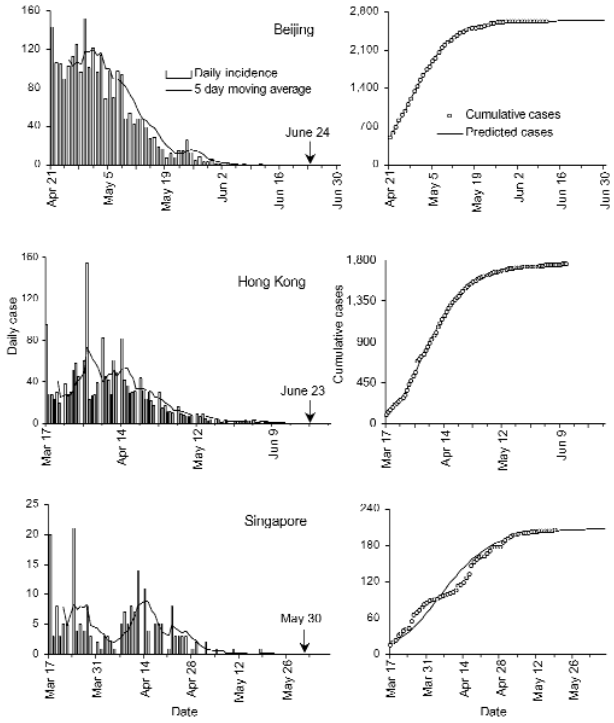


Figure. Epidemiologic depiction of epidemic of severe acute respiratory syndrome (SARS) in Beijing, Hong Kong, and Singapore. The number of daily confirmed SARS cases and 5-day moving average are represented by the left graphs. The observed and predicted cumulative cases since April 21, 2003 (Beijing), and March 17, 2003 (Hong Kong and Singapore), are shown in the right graphs. The modeling used case incidence data up to May 14, 2003. The arrow indicates the date that the World Health Organization removed the locality from the list of areas with local transmission.

## Conclusions

The association between cumulative number of cases and time is well described by the Richards model (Figure). For the three localities, highly significant correlations between observed and predicted incidence were found (adjusted $r^2 > 0.98$, $p < 0.01$ for goodness-of-fit of the model) (8). The maximum predicted cumulative incidence, *K,* was estimated to be 2,595 for Beijing, 1,748 for Hong Kong, and 207 for Singapore (Table). We estimated the 95% confidence interval (CI) of the predicted incidence and time for the epidemic to cease by inverse prediction based on the relationship $Log\ ((K/S)^{\alpha}-1)=rt_m-rt$ (8). If we assume that an epidemic is over when no new cases occur in 3 consecutive months, the earliest time for the end of the SARS epidemic, if intervention measures continued and no cases were imported, was estimated to be June 27, 2003, in Beijing; June 29, 2003, in Hong Kong; and May 28, 2003, in Singapore (see Table for 95% CI). Using 8.4 days as the generation time of a SARS infection, as estimated from the mean serial interval between the time from onset of symptoms in index patient to onset of symptoms in secondary case-patient in Singapore (9), we estimated the basic reproductive number of SARS infections, $R_0$, to be 2.7, 2.1, and 3.8 in Singapore, Hong Kong, and Beijing, respectively. The higher $R_0$ value in Beijing likely resulted from delays in exercising effective control measures. The $R_0$ estimates for Singapore and Hong Kong, when the Richards model and SARS case incidence data through May 14, 2003 were used, were similar to those based on stochastic models (9,10).

The transmission mechanism of the coronavirus that causes SARS and the epidemiologic determinants of spread of the virus are poorly understood (2). Our predictions were based on the trend analysis, assuming effective intervention measures would continue in the three cities. Predicting SARS dynamics on the basis of data from early in the epidemic could have lead to untenable conclusions (11); however, we found that the SARS epidemic in Hong Kong and Singapore in May 2003, was not in an early stage. The case data in these two localities clearly indicated S-shaped dynamics. Assuming SARS dynamics in Beijing would follow a similar pattern, we used the Richards model to predict that the SARS epidemic in Beijing would end by late June 2003. This prediction, made on May 21, 2003, was based on the trend analysis and assumed that effective intervention measures would continue.

On May 30, June 23, and June 24, 2003, respectively, WHO removed Singapore, Hong Kong, and Beijing from the list of areas with local transmission (12–14). As of July 10, a total of 8,436 SARS cases had been reported in 29 countries worldwide (15). The actual cumulative SARS cases were 206 for Singapore; 1,755 for Hong Kong; and 2,631 for Beijing (5,15). The observed total SARS incidence was within our predicted 95% CI for all three local-

Table. Predicted epidemic cessation date and maximum number of cases severe acute respiratory syndrome

| Locality | Parameter estimation[a] | | | Maximum no. of cases (95% CI)[b] | Epidemic cessation date (95% CI) |
|---|---|---|---|---|---|
| | $t_m$ | $r$ | $\alpha$ | | |
| Beijing | 8.94 | 0.16 | 1.00 | 2,595 (2,541 to 2,649) | June 27, 2003 (June 14 – July 10) |
| Hong Kong | 6.11 | 0.09 | 2.94 | 1,748 (1,619 to 1,777) | June 29, 2003 (June 14 – July 14) |
| Singapore | 14.50 | 0.12 | 1.51 | 207 (191 to 223) | May 28, 2003 (May 20 – June 5) |

[a]$t_m$, the inflection point of the growth model; $r$, the intrinsic growth rate; $\alpha$, the measurement of the extent of deviation of S-shaped dynamics from the classic logistic growth curve.
[b]CI, confidence interval.

ities (Table). The error rate (the difference between actual and predicted cumulative incidence divided by actual incidence) is 0.5%, 0.4%, and 1.4% for Singapore, Hong Kong, and Beijing, respectively.

The last probable SARS cases were reported on May 18 for Singapore, June 12 for Hong Kong, and June 11 for Beijing. The predicted SARS cessation date was later than the date the last probable SARS case was reported for all three cities but very close to the lower limit of the 95% CI (Table). Our results suggest that the simple Richards model describes well the SARS case incidence dynamics (under effective control measures) in Singapore, Hong Kong, and Beijing.

### Acknowledgment

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Dr. Zhou is a senior research scientist at the State University of New York at Buffalo. His research interests are the ecology and epidemiology of infectious diseases.

Dr. Yan is an associate professor of biological sciences at SUNY Buffalo; his research focuses on the ecology and genetics of infectious diseases.

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Address for correspondence: Guofa Zhou, Department of Biological Sciences, State University of New York at Buffalo, Buffalo, NY 14260, USA; fax: 716-645-2975; email: gzhou2@buffalo.edu

# Age and Variant Creutzfeldt-Jakob Disease

**Peter Bacchetti***

The young and stable median age of those who die of variant Creutzfeldt-Jakob disease has been attributed to age-dependent infection rates. This analysis shows that an influence of age on risk for death after infection better explains age patterns, suggesting that biologic factors peaking in the third decade of life may hasten disease.

The epidemic of variant Creutzfeldt-Jakob disease (vCJD) in Great Britain is now thought to be caused by the same prion responsible for bovine spongiform encephalopathy (BSE) in cattle (1). A striking feature of the human epidemic has been the young age of most patients and the lack of any trend toward older ages in patients dying later in the epidemic. Investigations of this stability have agreed that age must influence risk for infection (2,3), and studies projecting future numbers of cases have assumed that age only influences infection risk and does not influence risk for disease after infection (3–5). By extending previous methods to model age as a time-dependent covariate, I show here that the stable age distribution over time is in fact better explained by an influence of age on risk for disease after infection.

As was done in previous studies (2–5), I used methods to exploit the relation among date of infection, incubation time, and date of disease. The incubation period is defined as the time from infection to disease, which can be onset, diagnosis, or death. I focused on death from vCJD because no measurement error exists in the date of death, and ascertainment delay is less of an issue for this than for the date of disease onset. These methods were developed extensively for analyzing the HIV epidemic, both for estimating past infection rates, assuming a known incubation period distribution (6,7), and for estimating incubation, assuming a known infection pattern (8,9). To match the last approach, I assumed that the shape over time of the infection hazard (the risk for infection among uninfected persons) is determined by what is known about the BSE epidemic (10) and that the scale of the infection hazard is large enough so that the total number of infections (approximately 1.2 million) is much larger than the number of deaths to date. (This large number of infections implies that risk of developing disease after infection must be very low, so most of those infected will die of other causes, and disease will never develop.) I assumed that age has a multiplicative effect on the risk for infection or on the risk for disease after infection, corresponding to the proportional hazards assumption (11) frequently used in survival analysis. Detailed statistical methods are described in an online Appendix (available from: URL: http://www.cdc.gov/ncidod/EID/vol9no12/03-0361-app.htm).

Using the 121 reported deaths from 1995 to the end of 2002, I obtained similar shapes for the estimated influence of age on risk for infection (Figure a) or on risk for death (Figure b), with both showing strong peaks. The model of Figure b, where age influenced only risk for death, had a log likelihood that was better by 1.91 than the model of Figure a, where age influenced only risk for infection. A simulation test using data generated under the model of Figure a found differences this large 75 times in 2,000 runs ($p = 0.038$), indicating that the data are more compatible with an influence of age on risk for death. This age model appears to better explain the observed stability of ages at death over time. A simple regression of age at death on quarter of death estimates that the mean age has remained nearly constant at around 29 years, with an average increase of 26 days for each year of the epidemic. (A robust regression [12] of age at death on quarter of death found an increase of 20 days per year, nearly identical to the 26 days found by ordinary least squares regression.) The model in Figure b matched this finding with an estimated overall increase in mean age of 34 days per year, but the model in Figure a predicts an increase of 214 days per year.

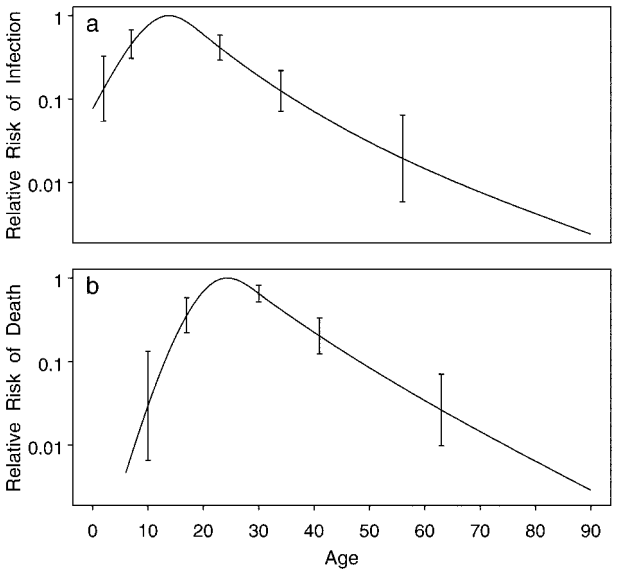


Figure. Estimated influence of age on a) risk for infection with the variant Creutzfeldt-Jakob disease (vCJD) agent and b) risk for death from vCJD after infection. Vertical bars are pointwise 95% confidence intervals for selected ages.

*University of California, San Francisco, California, USA

The shapes in the Figure contrast with those assumed by previous studies. One study assumed constant infection risk up to age 15, which is ruled out by the confidence intervals shown on the left in Figure a (3). Another study modeled the influence of age on both risk for infection and risk for death from vCJD after infection, but it imposed a mathematical form for the influence on risk for death that can only be monotonic, an assumption incompatible with the peaked shape in Figure b (2). That study's finding that age must influence risk for infection rather than only influencing risk for death is therefore suspect. In addition, a later analysis (13) claiming to confirm the impact of age on risk for infection did so only by assuming that age had no influence on subsequent risk for death.

In the Figure b model, younger persons' risks for death increase as they age (moving up the left slope), while older persons' risks decrease (moving down the right slope). Therefore, the highest risk moves toward younger and younger age cohorts over time, counteracting the aging of the entire infected population and thereby matching the observed stability in ages at death. Nothing counteracts the aging of the infected population if age is assumed to influence only risk for infection. This finding and reasoning contrast with an argument (3) that an influence of age on risk for death would result in a shift toward older cases later in the epidemic because most younger persons would have died earlier. This argument relies on the unstated assumption that the total number of infections is small enough that cases to date constitute a substantial fraction of the total infected in younger age cohorts. I have considered here the case that many more were infected. Even if the unstated assumption were true, this finding would not explain the observed stability because aging of the entire infected cohort should still produce an upward trend.

The estimates in the Figure assume that total infections were much greater than deaths reported to date but not so large that the pool of >20 million persons with the susceptible genotype became noticeably depleted before measures were implemented at the end of 1989 to keep contaminated beef out of the human food supply. I evaluated such a scenario, with most susceptible persons infected by 1989, and found even stronger evidence in favor of an influence of age on risk for death rather than risk for infection. The log likelihood was better by 3.13, and only 12 of 2,000 iterations in a simulation test produced a difference this large ($p = 0.006$).

The reasons for the age distribution of vCJD cases and its stability over time remain unclear, and epidemiologic analyses can provide limited insight. Previous assertions that age must influence risk for infection and that age does not influence development of disease may have been incorrect. Our findings suggest that the possibility should not be discounted that biologic factors peaking in the third decade of life may promote vCJD prion replication and consequent development of disease.

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Dr. Bacchetti is a professor of biostatistics at the University of California at San Francisco. His research interests include analysis of incomplete data, with particular emphasis on infectious diseases.

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Address for correspondence: Peter Bacchetti, Department of Epidemiology and Biostatistics, University of California, San Francisco, CA 94143-0560, USA; fax: +1 415-476-6014; email: peter@biostat.ucsf.edu

# Noninvasive Method for Monitoring *Pneumocystis carinii* Pneumonia

**Michael J. Linke,* Sandy Rebholz,† Margaret Collins,† Reiko Tanaka,† and Melanie T. Cushion*†**

The progression of *Pneumocystis carinii* pneumonia was temporally monitored and quantified by real-time polymerase chain reaction of *P. carinii*–specific DNA in oral swabs and lung homogenates from infected rats. DNA levels correlated with the number of *P. carinii* organisms in the rats' lungs, as enumerated by microscopic methods. This report is the first of a noninvasive, antemortem method that can be used to monitor infection in a host over time.

*Pneumocystis* pneumonia remains a leading opportunistic infection associated with AIDS patients, even in the era of highly active antiretroviral therapy (1). In developing countries, the incidence of infection has increased dramatically, with mortality rates ranging from 20% to 80% (2). An important limitation in its clinical management has been the inability to evaluate therapeutic response or to temporally measure the organism numbers because of the absence of an in vitro culture system. Our laboratory recently showed that the presence of *Pneumocystis carinii*–specific amplicons obtained from swabs of the oral cavities of nonimmunocompromised adult rats *(Rattus norvegicus)* was predictive of the development of *P. carinii* pneumonia after corticosteroid-induced immunosuppression (3). In the present study, we applied the oral swab technique in combination with quantification of organism-specific DNA using real-time polymerase chain reaction (PCR) to monitor the progression of infection in the rat model.

## The Study

Thirty-two male Long Evans rats (140–160 g) known to harbor *P. carinii* were obtained from Room 004 at the Cincinnati Veterinary Medical Unit (4). All rats produced *P. carinii* amplicons from initial oral swab samples taken before immunosuppression. After sampling, 8 of the 32 rats were euthanized and their lungs were removed and processed as described below. The remaining 24 rats were removed from the room and individually caged under barrier conditions, as described previously (3), to prevent transmission of infection that might occur between cage mates or from the environment. Barrier conditions consisted of the following: microisolator tops for each shoebox cage, which was then housed within a BioBubble (The Colorado Clean Room Company, Fort Collins, CO); autoclaved water, into which a sterile solution of cephadrine (Velosef; E.R. Squibb and Sons, Inc., Princeton, NJ) was injected for a final concentration of 0.200 mg/mL; autoclaved cages, bedding, and tops; and irradiated Lab Chow (Tekmar Irradiated Lab Chow, Harlan Industries, Indianapolis, IN). To provoke *P. carinii* pneumonia, 4 mg/kg of methylprednisolone acetate (Depo Medrol; The Upjohn Co., Kalamazoo, MI) was administered to the rats weekly for 10 weeks. At 4 and 7 weeks, swab samples were obtained from groups of eight rats; the rats were then euthanized. Their lungs were removed for quantification by microscopic enumeration of organism nuclei expressed as log nuclei/mL (5) and real-time PCR analysis under aseptic conditions. Six rats survived the 10 weeks of immunosuppression and were processed in an identical manner.

DNA was extracted from the oral swabs (OS) and lung homogenate (LH), as previously described (4). LH DNA was evaluated by spectrophotometric analysis at 260 and 280 nm. RC primers directed to a region of the mitochondrial large subunit rRNA (mtLSU) were used for amplification of *P. carinii*–specific DNA (6).

Real-time PCR was performed and results were analyzed on the iCycler iQ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA) under conditions of rapid melting at 95°C, annealing for 5 s at 55°C, and collection at 76°C for 10 s with 40 cycles of amplification. Five microliters of a 1/5 dilution of OS DNA or 2.5 ng of LH DNA were used in the reactions. Taq DNA (1.25 U) polymerase (Promega, Madison, WI) was used in the real-time PCR with a concentration of 2.5 mM $MgCl_2$ in 25-µL reactions. To monitor the accumulation of the products, 0.4 µL of 1/1,000 dilution of concentrated SYBR Green (Molecular Probes, Eugene, OR) was included in the reactions. All reactions were performed in triplicate. The mtLSU product was cloned into the TOPO-TA PCR cloning vector (InVitrogen, Carlsbad CA) (mtLSU-T-TA), quantified by spectrophotometry, and used to generate a standard curve. The cloned PCR product, ranging from 0.0005 pg to 0.5 pg per reaction, was used as a template; the threshold cycles ($C_T$s) of these reactions were then plotted against the log amount of plasmid per reaction in picograms.

*Veterans Affairs Medical Center, Cincinnati, Ohio, USA; and †University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

*P. carinii* DNA in the LH and OS samples was quantified by linear regression analysis of the $C_T$s relative to the standard curve (3). The concentration of *P. carinii* DNA in the LH and OS samples, determined from the standard curve in picograms, was converted to copies per milliliter by multiplying by the dilution factor based on the original concentration of DNA. The LH copies were log transformed and expressed as log copies per milliliter. The specificity of the reactions was verified by analysis of the product-melting curves and by gel electrophoresis. All products were of the expected size (137 bp) and produced a single peak with a $T_m$ of approximately 78°C.

Microscopic enumeration of nuclei of the lung homogenates was compared to real-time PCR lung homogenate results by using Tukey-Kramer Multiple Comparisons post-test to assess significance (InStat version 3; GraphPad Software, Inc., San Diego, CA). Pre- and postimmunosuppression OS samples were analyzed with the Mann-Whitney test (InStat v. 3). Spearman Rank Correlation was used to evaluate the correlation between microscopic enumeration and the real-time PCR output (Instat v.3).

To ensure accurate and reproducible results, the efficiency of the real-time PCR with the RC primer set was evaluated for each type of sample used in this study: mtLSU/T-TA, LH DNA, and OS DNA (Table 1). The exponential amplification and efficiency of the reactions were determined by evaluating the slope of the curve generated by plotting the log of known concentrations of template DNA vs. their $C_T$s (7). The RC primer set demonstrated acceptable levels of exponential amplification and efficiency with all three templates.

The organism numbers in lung tissue, quantified by microscopic enumeration, increased from log 4.69 after 4 weeks of immunosuppression to log 9.35 after 10 weeks of immunosuppression (Figure, A.). No organisms were detected in the lungs of the eight rats euthanized before the study began (level of sensitivity = ~10,000 nuclei per lung). The amount of *P. carinii*–specific DNA quantified by real-time PCR in the LH samples increased substantially from 0 to 7 weeks, with similar levels after 7 and 10 weeks of immunosuppression (Figure, B). Only one of eight rats euthanized at the initiation of the experiment produced quantifiable copies of *P. carinii*–specific DNA, with a level similar to those after 4 weeks of immunosuppression (data not shown). In every case, the postimmunosuppression OS taken from the rats at 4, 7, and 10 weeks

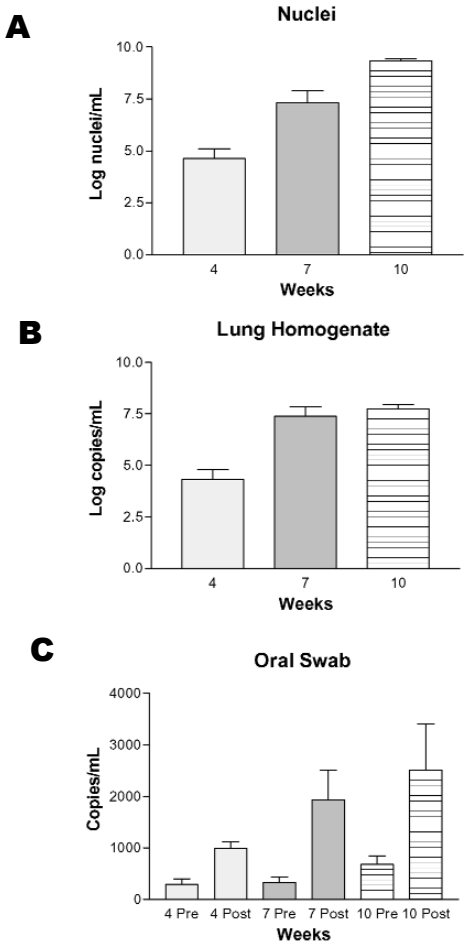


Figure. Progression of *Pneumocystis carinii* pneumonia measured by enumeration of organisms and real-time PCR of DNA extracted from lung homogenates and oral swabs. Panel A: Log *P. carinii* nuclei per mL of homogenized rat lung assessed by microscopic enumeration of lung homogenates; 4 wk vs. 7 wk, $p < 0.001$; 4 wk vs. 10 wk, $p < 0.001$; 7 wk vs. 10 wk, $p < 0.001$. Panel B: Log-transformed copies of *P. carinii*-specific DNA (mtLSU) per mL of lung homogenate; 4 wk vs. 7 wk, $p < 0.001$; 4 wk vs. 10 wk, $p < 0.001$; 7 wk vs. 10 wk, $p > 0.05$. Panel C: Copies of *P. carinii*-specific DNA/mL in oral swabs taken between immunosuppression (4, 7, and 10 wk preimmunosuppression) and at the time of euthanasia (4, 7, and 10 wk postimmunosuppression); pre- and post-4 wk, $p = 0.0006$; pre- and post-7 wk, $p = 0.0037$; pre- and post-10 wk, $p = 0.0221$. The ranges in value for the preimmunosuppression oral swabs at 4, 7, and 10 wk were, respectively, 165–487 copies/mL; 98–816 copies/mL; and 380–1,667 copies/mL. Ranges for the postimmunosuppression copies/mL after 4, 7, and 10 wk were 342–1,254; 517–5,256; and 637–6,279. (Number of rats in the 4- and 7- wk postimmunosuppression group = 8; number of rats in the 10-wk group = 6.)

Table 1. Efficiencies of the real-time PCR reactions[a]

| Template | Range | $r^2$ | Slope | Amplification | Efficiency |
|---|---|---|---|---|---|
| mtLSU | 0.5 to 0.0005 pgs/rx | 0.999 | -3.220 | 2.043 | 1.043 |
| LH | 12.5 to 1.25 x 10-5 ngs/rx | 0.966 | -3.570 | 1.906 | 0.906 |
| OS | Undiluted to 1:8 dilution | 0.973 | -3.328 | 1.997 | 0.997 |

[a]PCR, polymerase chain reaction; mtLSU, mitochondrial large ribosomal subunit RNA; LH, rat lung homogenate; OS, oral swab *P. carinii*–specific DNA; rx, reaction.

had significantly more *P. carinii*–specific DNA than the preimmunosuppression OS taken at the initiation of the study (Figure, C). The amount of *P. carinii*–specific DNA in the OS samples also increased over time (Figure, C). No significant correlation was found between the amount of *P. carinii* DNA detected in the preimmunosuppression OS samples and the amount in the postimmunosuppression OS samples, the lung homogenates, or nuclei number, suggesting that the rats had equivalent but low levels of organisms at the initiation of the study.

To determine the relationship between quantitation of *P. carinii* by real-time PCR and by microscopic enumeration, results were analyzed by Spearman rank correlation (Table 2). A significant correlation was found between both the amount of *P. carinii* DNA detected in the postimmunosuppression OS samples and in the LH versus the number of *P. carinii* nuclei. A significant correlation was also detected between the real-time PCR quantitation of *P. carinii* DNA in the OS and the LH.

## Conclusions

The combination of antemortem oral swab sampling and real-time PCR amplification and quantification reported here should be useful for the study of the *Pneumocystis* infections in other experimental models and provides a rationale for similar studies to be conducted in the clinical setting. Real-time PCR previously has been shown to be useful for quantitation of the level of infection in the lungs of infected rats and mice, but the studies were performed on postmortem samples or purified organisms (8,9) *P. jiroveci* DNA levels from oral washes, induced sputa, and bronchoalveolar lavage fluids from humans have been quantified by using various real-time PCR techniques (10–13) as well, but the findings were used for diagnosis, detection, or quantification and did not obtain samples from individual hosts over time. In our study, the levels of *P. carinii* DNA in the oral cavities of the rats were measured temporally and shown to correlate with the numbers of organisms in the lungs, establishing the oral swab real-time PCR technique as a surrogate means of following the progress of the infection. Successful application of this method to the human infection would enhance epidemiologic studies, permit sensitive and rapid assessment of therapeutic response, and allow basic biologic questions of carriage length and potential reservoirs to be addressed.

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Dr. Linke is a research microbiologist at the Veterans Affairs Medical Center in Cincinnati, Ohio. His major research interest is the role of the innate immune response in the prevention and clearance of *Pneumocystis* infection.

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Table 2. Comparisons of *Pneumocystis carinii*–specific DNA levels in pre- and postimmunosuppression samples, lung homogenates, and organism numbers in lung homogenates assessed by microscopic enumeration[a]

| Groups[b] | No. points | Spearman r | 95% Confidence interval | p value | Significant |
|---|---|---|---|---|---|
| LH Pc DNA vs. post-OS Pc DNA | 22 | 0.5576 | 0.1648 to 0.7978 | 0.0070 | Yes |
| LH Pc DNA vs. Pc nuclei | 22 | 0.9035 | 0.7731 to 0.9606 | <0.0001 | Yes |
| Post-OS Pc DNA vs. Pc Nuclei | 22 | 0.4636 | 0.0388 to 0.7465 | 0.0298 | Yes |
| Pre-OS Pc DNA vs. post-OS Pc DNA | 21 | 0.3707 | -0.0863 to 0.6988 | 0.0980 | No |
| Pre-OS Pc DNA vs Pc Nuclei | 21 | 0.4123 | -0.0374 to 0.7232 | 0.0633 | No |
| Pre-OS Pc DNA vs. LH Pc DNA | 21 | 0.2939 | -0.1712 to 0.6519 | 0.1960 | No |

[a]21 data points were included in these analyses because 2 rats from the 10-wk group died and 1 preimmunosuppression oral swab sample in the 7-wk group was lost in processing.
[b]Pre-OS Pc DNA, *P. carinii* –specific DNA from oral swabs taken prior to immunosuppression; post-OS Pc DNA, *P. carinii*–specific DNA from oral swabs taken at the time of euthanasia; LH Pc DNA, *P. carinii*–specific DNA from lung homogenates of rats at the 3 different time points; log Pc Nuclei, *P. carinii* organism number assessed by microscopic enumeration.

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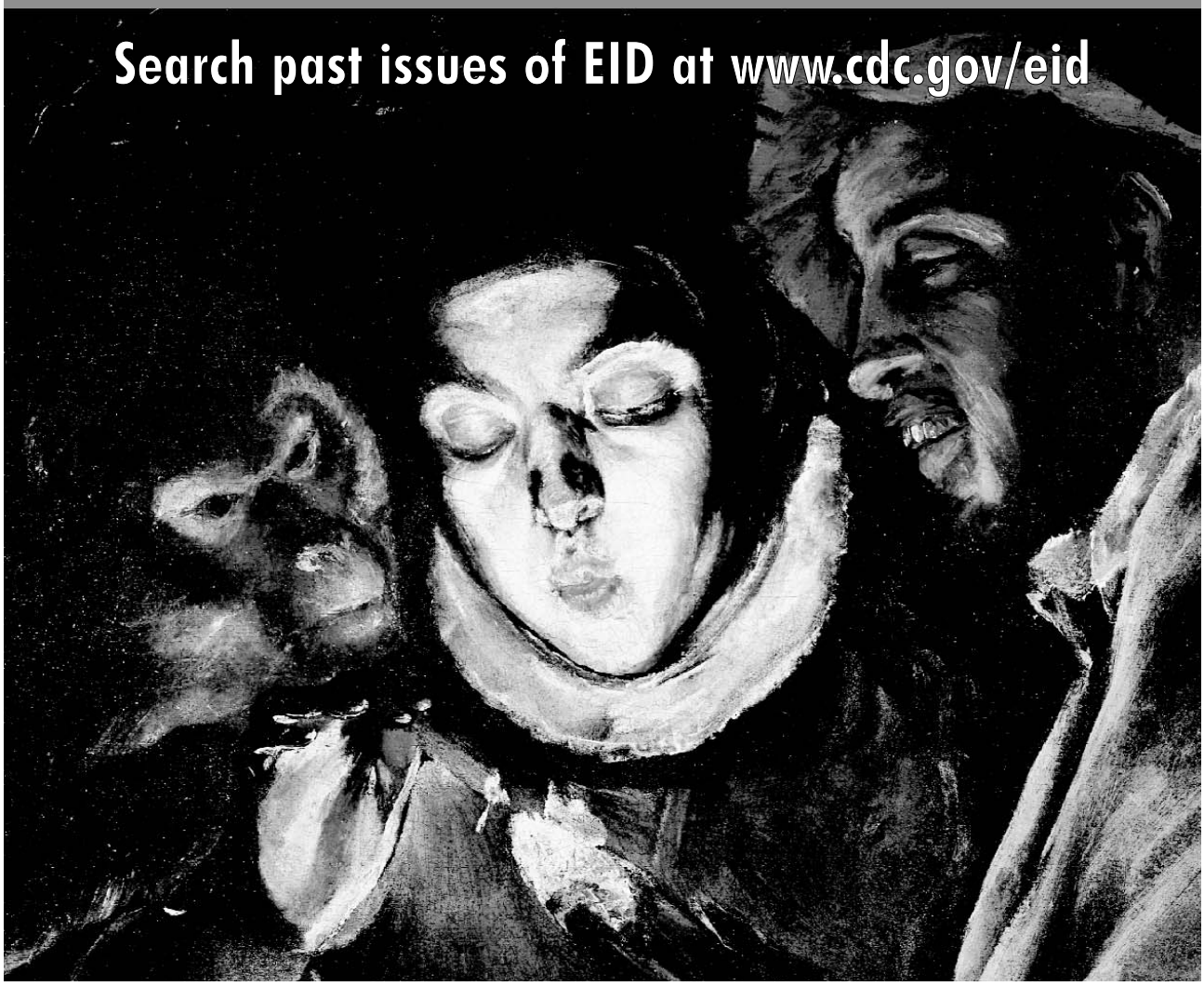
Address for correspondence: Melanie T. Cushion, Department of Internal Medicine, Division of Infectious Diseases, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0560, USA; fax: 513-475-6415; email: Melanie.Cushion@med.va.gov





# Visceral Leishmaniasis Treatment, Italy

**Luigi Gradoni,* Marina Gramiccia,* and Aldo Scalone***

First-line drug treatment was recorded in 573 immunocompetent patients with visceral leishmaniasis in Italy. In the past 12 years, the proportion of antimonial treatments decreased from 100% to 2.8%, while the proportion of amphotericin B treatments increased from 0% to 97.2%. The countrywide change in therapy is a response to both disease reemergence and increasing antimonial failure.

Zoonotic visceral leishmaniasis is a life-threatening disease caused by the multiplication of the protozoan parasite *Leishmania infantum* in the phagocytes of the reticuloendothelial system. Infections are widespread in the Mediterranean subregion, where the parasite is transmitted in summer by the bites of phlebotomine sand flies, and canids serve as reservoir hosts (1).

In the first half of the 20th century, visceral leishmaniasis was a typical infantile syndrome in Italy with high incidence in southern regions and islands. After World War II, the incidence dropped to 10 to 20 cases per year for 4 decades; the disease reemerged with approximately 200 cases in 2000 and 2001 (Figure 1). This trend can be explained by the following: 1) the appearance of cases in immunocompetent adults that might be attributable to a general decrease in acquired immunity after the reduction of the phlebotomine-vector populations, determined by the massive antimosquito insecticide campaigns for malaria eradication 50 years ago (2); 2) the spreading of the disease from traditional areas of transmission to new stable foci in central and northern regions of Italy, as evidenced by recent colonization of these areas by sand flies and by increased *Leishmania* diffusion and prevalence among the canine reservoir (3); and 3) the occurrence of *Leishmania* infections in immunosuppressed persons, such as those co-infected with HIV (4). Incidence of visceral leishmaniasis in these patients, however, has recently decreased after the introduction of highly active antiretroviral therapy (Figure 1) (5).

Since the 1940s through 1990, meglumine antimoniate has been the only first-line drug for visceral leishmaniasis treatment in Italy (6). From 1991 through 1994, a total of 90 patients of all ages, representing one third of all immunocompetent visceral leishmaniasis case-patients reported in Italy during that period, were enrolled in clinical trials of liposomal amphotericin B (L-AmB), which led to a novel, safe, short course of visceral leishmaniasis treatment as an alternative to meglumine antimoniate (7,8). In the same period, other lipid-associated AmB drugs were registered in Italy for the treatment of fungal infections, i.e., AmB colloidal dispersion (ABCD) and AmB lipid complex (ABLC). Because no official policy exists for visceral leishmaniasis therapy in Italy (physicians can prescribe any registered drug under their own responsibility) and information on drug regimens used is not included in visceral leishmaniasis case reports, we aimed to assess whether changes have occurred, and to what extent, in first-line drug regimens adopted in Italy after lipid-associated AmB was introduced into clinical practice.

## The Study

A retrospective analysis was performed on data collected at the Unit of Protozoology of Istituto Superiore di Sanità, Rome, the main reference center for visceral leishmaniasis surveillance in Italy. Diagnosis of visceral leishmaniasis in patients with clinically suspected cases was routinely performed on serum and bone marrow aspirate samples sent by hospitals, mainly from pediatrics, internal medicine, and infectious diseases wards, from throughout the country. If visceral leishmaniasis was confirmed, relevant information on patients was recorded, which included drug regimens used and posttherapy results. Two datasets were analyzed: the first included information from patients in whom leishmaniasis was diagnosed from 1986 to 1990, i.e., before the mass enrollment of patients in the aforementioned study on L-AmB; the second from patients in whom leishmaniasis was diagnosed from 1995 to 2001, i.e., after that study. Immunosuppressed patients (e.g., HIV co-infected persons or transplant recipients), who usually respond poorly to antileishmanial treatments, were not included in our analysis. Fisher exact test was used for comparisons.

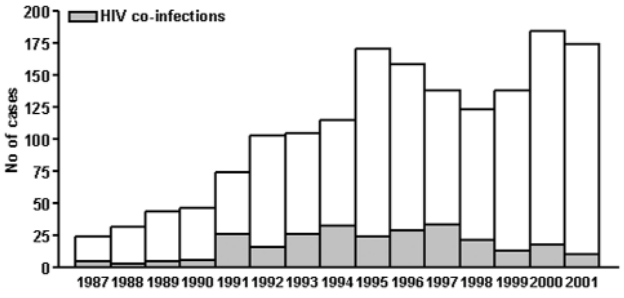



Figure 1. Reemergence of zoonotic visceral leishmaniasis in Italy: human cases recorded from 1987 through 2001 by passive reports and active surveillance.

*Istituto Superiore di Sanità, Rome, Italy

For the 1986–1990 period, we recorded treatments used for 40 patients in 22 hospitals, representing 29.2% of 137 immunocompetent persons with visceral leishmaniasis. Fourteen (35.0%) were children <14 years of age. As expected, all patients were treated with meglumine antimoniate, given at the standard dose of 20 mg pentavalent antimony ($Sb^v$)/kg/day for 3 to 4 weeks (6), either alone (37 patients) or in combination with allopurinol at the daily dose of 15 mg/kg (3 patients). Two patients treated with meglumine antimoniate alone (5.4%) had a visceral leishmaniasis relapse within 6 months from treatment and have been retreated successfully with meglumine antimoniate in combination with allopurinol.

For the 1995–2001 period, we recorded treatment information for 533 patients, representing a large proportion (56.4%; annual range 43.3% to 69.1%) of 945 immunocompetent visceral leishmaniasis patients. About half were children (267; annual range in proportion 42.1% to 64.8%). Every year, patients were referred by 19 to 42 hospitals, with a range of 1 to 30 patients per hospital. Drug regimens recorded are shown in the Table and summarized in Figure 2. Meglumine antimoniate was the first-line drug used in 158 patients (29.6%) at the $Sb^v$ dosages noted previously; 6 also received allopurinol (the drug combination was used until 1997). The proportion of meglumine antimoniate-treated patients has steadily decreased from 55.9% in 1995 to 2.8% in 2001. AmB drugs have been the only alternative drugs used in the remaining 375 patients (70.4%). Of those patients, L-AmB accounted for most regimens (348, 92.8%); this drug was administered to both children and adults at the standard dose of 3 mg/kg/day for 5 consecutive days plus an additional 3 mg/kg dose on day 10 (7). Slight variations from this regimen (e.g., 3 mg/kg/day for 7–10 consecutive days) were recorded in some case-patients. ABCD and ABLC were given only to adult patients (21 and 3 cases, respectively) both at the dosage of 2 mg/kg/day for 7 days. Thus, lipid-associated AmB was used to treat 372 patients (69.8%). Finally, three adult patients were treated with the conventional AmB deoxycholate formulation (dosages unreported). The proportion of patients treated with any AmB-based drugs increased from 44.1% in 1995 to 97.2% in 2001.

We recorded the failure of drug therapy in 16 (10.1%) of 158 patients treated with meglumine antimoniate, equally distributed in children and adults and with no association with particular geographic location. Five patients showed primary unresponsiveness or experienced acute toxicity, which required suspension from treatment, while 11 patients who responded initially to treatment had a relapse after a variable period of time (range 3–11 months). All patients were successfully retreated with the standard L-AmB regimen. Altogether, the rate of meglumine antimoniate failures recorded in 1995 to 2001 did not differ significantly from that of the failures in 1986 to 1990. However, the rate significantly increased in recent years, from 3 (5.3%) of 57 in 1995 to 4 (36.4%) of 11 in 2000 ($p = 0.01$). Drug treatment was unsuccessful in 12 (3.2%) of 375 AmB-treated patients, in 2 patients the infection was unresponsive, and 10 patients had a relapse at 3 to 10 months. This rate was significantly lower than the meglumine antimoniate failure rate ($p = 0.002$). Eight of 10 L-AmB treatments failed in children from different geographic locations. All AmB treatment failures but one (retreated with meglumine antimoniate) were successfully retreated with a high-dose L-AmB regimen of 3 mg/kg/day for 10 consecutive days.

## Conclusions

A range of treatment options exists in visceral leishmaniasis, which include two pentavalent antimonials (meglu-

Table. First-line drugs used for treatment of visceral leishmaniasis in 533 immunocompetent patients in Italy and drug treatment failures recorded[a]

| Y | MA (%) | L-AmB | ABCD | ABLC | dAmB | Any AmB drugs (%) |
|---|---|---|---|---|---|---|
| 1995 | 57 (55.9) | 45 | 0 | 0 | 0 | 45 (44.1) |
| | 3 R | 1 R | | | | 1 R |
| 1996 | 35 (50.0) | 35 | 0 | 0 | 0 | 35 (50.0) |
| | 2 R | 1 R | | | | 1 R |
| 1997 | 26 (39.4) | 34 | 5 | 0 | 1 | 40 (60.6) |
| | 3 U, 1 R | 2 R | | | | 2 R |
| 1998 | 14 (28.0) | 32 | 4 | 0 | 0 | 36 (72.0) |
| | 1 R | 1 R | | | | 1 R |
| 1999 | 13 (21.0) | 45 | 2 | 1 | 1 | 49 (79.0) |
| | 1 U, 1 R | 2 R | | | | 2 R |
| 2000 | 11 (9.8) | 89 | 10 | 1 | 1 | 101 (90.2) |
| | 1 U, 3 R | 1 U, 1 R | 1 U | 1 R | | 2 U, 2 R |
| 2001 | 2 (2.8) | 68 | 0 | 1 | 0 | 69 (97.2) |
| | | 1 R | | | | 1 R |
| Total | 158 (29.6) | 348 | 21 | 3 | 3 | 375 (70.4) |
| | 5 U, 11 R | 1 U, 9 R | 1 U | 1 R | | 2 U, 10 R |

[a]MA, meglumine antimoniate; L-AmB, liposomal amphotericin B; ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B lipid complex; dAmB, amphotericin B desoxycholate; U, unresponsiveness and/or acute toxicity; R, relapse.

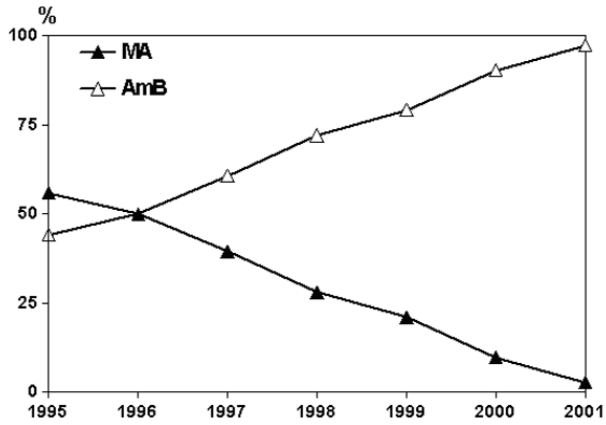



Figure 2. Annual proportion of immunocompetent patients with visceral leishmaniasis treated with meglumine antimoniate (MA) or amphotericin B (AmB) in the period 1995–2001.

mine antimoniate and sodium stibogluconate), four formulations of AmB, aminosidine (paromomycin), pentamidine, and the new oral agent miltefosine (9,10). For the clinician, the choice of treatment depends on several factors, such as the clinical features of the disease, as well as drug safety, efficacy, and cost. In the absence of any official drug policy for visceral leishmaniasis in Italy and in consideration of the large sample of patients surveyed, our study may be the first observational investigation on visceral leishmaniasis therapy at the national level from 1986 through 2001.

Results have shown a countrywide change in therapy over the period considered. Even though the change was relatively gradual over a 16-year period, a traditionally effective drug (meglumine antimoniate) has been almost fully replaced by a new compound, L-AmB, in an epidemiologic context of disease reemergence. Possible explanations for this change include the following: 1) mild or severe adverse reactions (e.g., pancreatitis, cardiac abnormalities) are commonly seen in meglumine antimoniate–treated patients, especially in adults, when the recommended dosages are increased even slightly. A recent investigation in Sicily showed that the antimony-associated death rate was 7% among HIV-negative adults with or without underlying diseases (11). On the other hand, the toxicity of lipid-associated AmB drugs, especially L-AmB, was negligible in all categories of patients at the dosages used for visceral leishmaniasis therapy (9). 2) The efficacy of meglumine antimoniate for the treatment of Mediterranean visceral leishmaniasis has been high (approximately 95%) for >50 years. However, in the past few years, meglumine antimoniate treatment failures have increased in visceral leishmaniasis patients from southern Europe. This treatment failure could be attributable to the widespread use of meglumine antimoniate in treating infected dogs, which may have caused the spread of *L. infantum* strains less susceptible to antimony (12–14). Efficacy of AmB drugs is very high and, so far, decreased *Leishmania* susceptibility to this compound (AmB is rarely used in veterinary practice) has not been indicated. 3) Although L-AmB and other lipidic formulations of AmB are 30- to 50-fold more expensive than meglumine antimoniate for visceral leishmaniasis therapy at the dosages reported above, in Western countries most of the costs of treating visceral leishmaniasis are inpatient hospitalization expenses rather than drug costs. Therefore, short courses of 6 to 7 days, as required for L-AmB, ABCD, or ABLC (9), are highly cost-effective if compared with 21- to 28-day courses needed for meglumine antimoniate treatment.

Dr. Gradoni is the head of the Protozoology Unit in the Parasitology Department of Istituto Superiore di Sanità in Rome. His main research interests are in the epidemiology and control of leishmaniases.

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Address for correspondence: Luigi Gradoni, Laboratorio di Parassitologia, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy; fax: 39-06-4938-7065; email: gradoni@iss.it

# Ciprofloxacin Treatment Failure in Typhoid Fever Case, Pakistan

**Tariq Butt,* Rifat Nadeem Ahmad,* Abid Mahmood,* and Sabeen Zaidi***

We report a case of ciprofloxacin treatment failure in a typhoid fever patient at a tertiary care hospital in Rawalpindi, Pakistan. This case shows not only the emergence of fluoroquinolone resistance in typhoid salmonellae but also the inadequacy of the current laboratory guidelines for detection of this resistance.

Typhoid fever is a major health concern in the developing world; >16 million new cases occur worldwide annually, resulting in approximately 600,000 deaths per year. The last two decades have seen the emergence and spread of multidrug resistance against the conventional antityphoid drugs (chloramphenicol, co-trimoxazole, and ampicillin) among the typhoid salmonellae, especially in South and Southeast Asia, including Pakistan. These developments had left fluoroquinolones as the antimicrobial agents of choice for the treatment of typhoid fever (1). Fluoroquinolone resistance is being reported with increasing frequency from all over the world (1–5). We report ciprofloxacin treatment failure in a case of typhoid fever.

## Case Report

A previously healthy 14-year-old boy from Rawalpindi, Pakistan, was admitted in July 2002 to Combined Military Hospital, Rawalpindi, with a 7-day history of a high fever (>38°C) and vomiting. He had relative bradycardia (heart rate 84 bpm) and a soft palpable spleen. His total leukocyte count was 3 x $10^9$/L. Malarial parasites were not seen on examination of thin and thick smears of peripheral blood. The results of a routine urinalysis and chest radiographs were normal. A blood Widal test showed a titer of 320 against "O" (somatic) antigen of *Salmonella enterica* serovar Typhi. Blood culture yielded the growth of *Salmonella* Typhi. The isolate was found to be resistant to the conventional antityphoid drugs by using modified Kirby-Bauer disk diffusion technique according to the criteria of the National Committee for Clinical Laboratory Standards (NCCLS) (6). The disks of antimicrobial drugs used were chloramphenicol (30 μg), co-trimoxazole (1.25/23.75 μg), ampicillin (10 μg), ciprofloxacin (5 μg), and ceftriaxone (30 μg). The isolate appeared susceptible to ciprofloxacin and ceftriaxone.

The patient was given ciprofloxacin, 500 mg, every 12 hours, orally, on admission but remained febrile after 3 days of treatment. When the blood culture report was received, and in view of the susceptibility pattern, intravenous ciprofloxacin, 200 mg every 12 hours, was administered. Despite 8 days of treatment, his fever did not resolve. The isolate was reviewed and the MIC of ciprofloxacin was determined by Kirby-Bauer broth dilution technique; it was 0.5 μg/mL; well below the NCCLS recommended break point value of 1 μg/mL (7). However, in light of the treatment failure with ciprofloxacin, intravenous ceftriaxone, 1 g every 12 hours, was administered, and the patient responded within 3 days.

## Conclusions

This case highlights two developments: first, the increasing incidence of reduced susceptibility and resistance of typhoid salmonellae against fluoroquinolones, and second, the inadequacy of the present laboratory guidelines for detecting fluoroquinolone resistance in typhoid salmonellae. The first case of ciprofloxacin-resistant typhoid fever was reported in 1992 in the United Kingdom (8), and the first case of fluoroquinolone treatment failure in typhoid fever in Pakistan was reported in 1993 (9). Similar cases have been reported from several other countries (1–5). Selective pressure on the bacterial population by uncontrolled use of these antimicrobial drugs has likely led to the emergence of this resistance (2), which has been attributed to a single point mutation in the quinolone-resistance–determining region (QRDR) of the topoisomerase gene *gyrA* (1,2,5,10). However, other mechanisms such as decreased permeability and active efflux of the antimicrobial agent may also be involved (10).

The inadequacy of the current in vitro antimicrobial susceptibility testing for detecting fluoroquinolone treatment failure in typhoid fever is apparent in this case. According to NCCLS guidelines, Enterobacteriaceae (including typhoid salmonellae) are susceptible to the MIC of <1 μg/mL of ciprofloxacin, while resistant to the MIC of >4 μg/mL (7). But in our case-patient, treatment failed, even though the MIC was stated as 0.5 μg/mL. Similar observations have been made in other countries (2–5,11). Keeping in view this absence of correlation between MIC of fluoroquinolones and therapeutic response in typhoid fever, we recommended break point MIC values of ciprofloxacin in cases of typhoid salmonellae infection as follows: <0.125 μg/mL as susceptible, 0.125 μg/mL–1 μg/mL as reduced susceptibility, and >1 μg/mL as resistant. Determination of MIC may not be practicable in routine laboratory practice, particularly outside of a reference laboratory in developing countries. Also, disk diffusion

*Armed Forces Institute of Pathology, Rawalpindi, Pakistan

criteria with ciprofloxacin are inadequate to highlight these new recommended MICs, and detecting mutation in the QRDR of *gyrA* gene by polymerase chain reaction (PCR) would not be practical or cost-effective. Several authors have reported a correlation between resistance to nalidixic acid and reduced susceptibility to ciprofloxacin and other fluoroquinolones (2,11). Routine testing of resistance to nalidixic acid with a disk content of 30 µg can serve as a useful screening test for fluoroquinolone resistance (11). However, revision of the diagnostic criteria for detecting fluoroquinolone resistance in typhoid salmonellae is needed, particularly to validate clinically all the laboratory-based anecdotal studies. Even with adoption of the new recommended MICs of fluoroquinolones against typhoid salmonellae, MICs would have to be correlated with inhibition zone size by disk diffusion technique and the clinical response in infection with typhoid salmonellae depicting reduced susceptibility against quinolones.

To summarize, fluoroquinolones are the most effective antimicrobial agents for treating enteric fevers (1). Emergence of resistance against them is of major concern. The spread of this resistance would leave only the less effective (1,2), but more expensive, third-generation cephalosporins for treatment of typhoid. Fluoroquinolone resistance must be identified early, and these drugs must be used judiciously. Otherwise, society may be faced with the prospect of untreatable typhoid fever.

Dr. Butt is a consultant microbiologist and head of the Microbiology Department at the Armed Forces Institute of Pathology, Rawalpindi, Pakistan. His research interests include tuberculosis and enteric fevers.

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Address for correspondence: Brigadier Tariq Butt, Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan; fax: 92-51-9271247; email: tariqbutt24@yahoo.com

# Novel Lyssaviruses Isolated from Bats in Russia

**Alexandr D. Botvinkin,* Elena M. Poleschuk,† Ivan V. Kuzmin,†‡ Tatyana I. Borisova,* Suren V. Gazaryan,§ Pamela Yager,‡ and Charles E. Rupprecht‡**

Two new rabies-related viruses were discovered in Russia during 2002. Viruses were isolated from bats in Eastern Siberia near Baikal Lake and in the western Caucasus Mountains. After preliminary antigenic and genetic characterization, we found that both viruses should be considered as new putative lyssavirus genotypes.

Rabies is an acute, fatal encephalitis caused by lyssaviruses that are perpetuated in reservoir mammals, principally certain carnivores and bats. Although the disease has been known among carnivores, such as dogs, for centuries, the paradigm of rabies in bats has been appreciated fully only over the past 50 years (1–3). Recent findings of bat lyssaviruses throughout the world have prompted a taxonomic reconsideration of the *Lyssavirus* genus, family *Rhabdoviridae*. To date, besides their occurrence in the Americas, Africa, and Australia, at least four additional bat lyssaviruses have been identified in Eurasia (4–7). One of these has been reported from Russia, a "Duvenhage-like" virus isolated from a patient who died in 1985 after being bitten by a bat at a site near the Urkrainian border (8). We describe the isolation and preliminary identification of two new bat lyssaviruses discovered in Russia.

## The Study

During preliminary infectious disease surveys, bats were obtained randomly at different locations by hand at roosts and from mist netting at cave entrances and at routes of nocturnal foraging. From 1979 to 2002, a total of 210 bats were collected in the Baikal Lake region, including 98 *Vespertilio murinus*, 3 *Myotis brandtii*, 55 *M. daubentonii*, 2 *M. iknnikovii*, 29 *Eptesicus nilssonii*, 22 *Plecotus auritus*, and 1 *Murina leucogaster*. In the Caucasus Mountains, 129 bats were collected during a field expedition in July 2002, including 6 *Rhinolophus ferrumequinum*, 10 *Myotis blythii*, 43 *M. daubentonii*, 4 *M. emarginatus*, 9 *Pipistrellus kuhlii*, 2 *P. pipistrellus*, 3 *Barbastella barbastellus*, 28 *Nyctalus noctula* and 24 *Miniopterus schreibersii*. After they were collected, the bats were euthanized by cervical disposition, and tissues were removed at necropsy.

In the laboratory, bat brain samples were screened as described (9) by the intracerebral mouse inoculation test (MIT) with 3- to 4-week-old inbred mice and by an enzyme-linked immunosorbent assay (ELISA) with polyclonal antirabies immune globulin. Brains of animals that died during MIT were subjected to the direct fluorescent antibody test (DFAT) with polyclonal antirabies fluorescein (FITC)-labeled immune globulins (Scientific-Research Veterinary Institute, Kazan, Russia) or FITC-labeled antirabies diagnostic conjugate (Centocor Inc., Malvern, PA).

If viral antigen was detected in mouse brain by the DFAT or ELISA, the agent was further characterized by antigenic typing by using panels of antinucleocapsid monoclonal antibodies (N-MAbs) of the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and N-MAbs 502-2 and 422-5 of the Wistar Institute (Philadelphia, PA) as described (10,11). For genetic typing, nucleic acid was extracted from infected brains, with amplification by reverse trancription-polymerase chain reaction (RT-PCR) and direct sequencing of the PCR products performed as described (7). Phylogenetic analysis of limited N gene sequences (400 bp from the amino-terminus) was performed by the neighbor joining method using MEGA software, version 2.1 for 1000 bootstrap replicates (12). The vesicular stomatitis virus nucleoprotein gene sequence (GenBank accession no AF473864) was used as an outgroup.

From the 339 bats examined, two lyssaviruses were isolated. In Eastern Siberia, an isolate (named Irkut virus) was obtained in the town of Irkutsk. A male Greater Tube-nosed Bat (*Murina leucogaster*) entered an apartment in September 2002. The bat was captured and maintained in captivity. The bat exhibited no abnormal behavior at first. After a few days, however, the bat became sluggish, rejected food and water, and died, approximately 10 days after capture, with signs of general exhaustion and weakness. An ELISA indicated that the bat's brain was strongly positive with antirabies immune globulin. The brain also demonstrated typical fluorescent intracytoplasmic inclusions by DFAT, with Russian (Kazan) and Centocor FITC-globulins. In the MIT, one mouse became sick and paralyzed; he was euthanized after 18 days of incubation. The mouse's brain was strongly positive for lyssavirus antigen by DFAT. During a second MIT intracerebral passage, the incubation period varied from 9 to 18 days. Intramuscular inoculation of mice with this virus was successful also, but susceptibility was less, producing a titer difference of log 4.2 MIC $LD_{50}$, than with the intracerebral route.

*Plague Control Research Institute of Siberia and the Far East, Irkutsk, Russia; †Research Institute for Natural Foci Infections, Omsk, Russia; ‡Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and §Krasnodar, Russia

A second isolate was obtained from the Caucasus (about 100 km southeast of the town of Krasnodar). All bats collected in this survey appeared healthy. No sick bats or carcasses were found in caves or other roosts. ELISAs of 129 bat brain samples gave negative results. The MIT produced one positive result, from the brain of a male Common Bent-winged Bat (*Miniopterus schreibersi*), captured during departure for nocturnal foraging at a cave entrance, together with 23 other males of the same species. Inoculated mice became paralyzed and died 9–13 days after intracerebral inoculation. Mouse brain impressions were DFAT-positive with either Russian (Kazan) or Centocor FITC-globulins. Intramuscular inoculation of 3-week-old mice was unsuccessful with at least log 5.7 MIC $LD_{50}$/0.05 mL. Based on the location, the virus was named West Caucasian bat virus (WCBV).

In antigenic typing, both viruses reacted with Wistar N-MAb 502-2, but only the African nonrabies lyssaviruses reacted with N-MAb 422-5 (Table). With CDC N-MAbs, the patterns obtained for Irkut virus were similar to those of Duvenhage and European bat lyssavirus, type 1 (EBLV-1), but distinguishable from both of them, whereas WCBV demonstrated unique patterns.

When phylogenetic analysis was performed, Irkut virus was recognized as a member of a cluster joining lyssavirus genotypes 4 and 5 (76% bootstrap support). However, the degree of diversity did not allow us to consider it a representative of one of these genotypes (Figure). WCBV was connected to the cluster of genotypes 2 and 3, but bootstrap support of this joining was insignificant (68%), illustrating that this virus is the most divergent member of the *Lyssavirus* genus examined to date. Further analysis of the

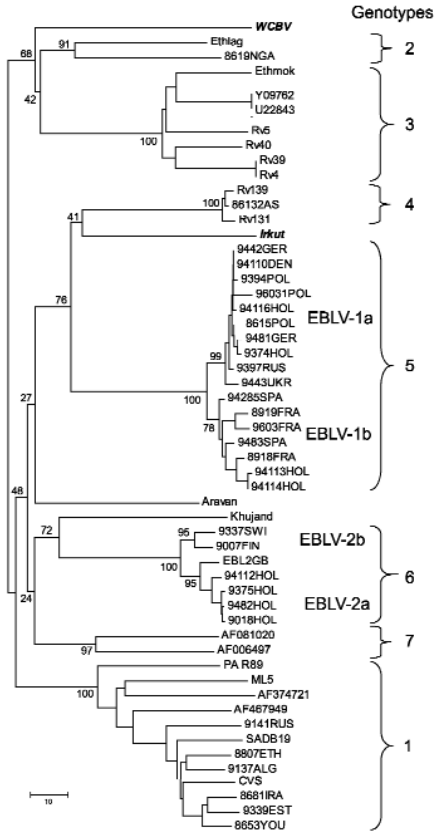


Figure. Neighbor-joining phylogenetic tree of the *Lyssavirus* genus, based on limited N gene sequences (400 bp from the amino terminus). Virus names are provided according to GenBank records, except for Ethmok and Ethlag. Subgroups "a" and "b" of EBLV-1 and EBLV-2 viruses are given according to Amengual et al.(5). Bootstrap values are presented for key nodes, and branch lengths are drawn to scale.

Table. Antigenic patterns of new bat virus isolates compared to other lyssaviruses by a panel of N-MAbs[a]

| | N-MAbs | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus | 3-1 | 8-2 | 11-1 | 15-2 | 22-3 | 23-4 | 24-1 | 24-10 | 52-1 | 52-2 | 61-1 | 62-4 | 71-2 | 97-3 | 97-11 | 141-1 | 143-1 | 146-3 | 164-2 | 502-2 | 422-5 |
| Irkut virus | + | - | + | - | + | o | - | + | + | + | - | - | - | - | - | + | - | - | - | + | - |
| WCBV | - | + | - | - | + | - | + | - | + | - | + | + | + | - | - | - | - | - | - | + | - |
| Lagos bat virus (variant 1)[b] | - | - | + | - | + | - | - | - | + | + | - | - | - | - | - | + | - | - | - | + | + |
| Lagos bat virus (variant 2)[b] | - | - | + | - | + | - | - | + | + | + | - | - | - | - | - | + | - | - | - | + | + |
| Mokola[b] | - | - | + | - | + | - | - | - | + | + | - | - | - | - | - | + | + | + | - | + | + |
| Duvenhage virus[b] | - | - | + | - | + | + | - | + | + | + | + | - | - | - | - | + | - | - | - | + | + |
| EBLV-1[b] | + | - | + | - | + | + | - | + | + | + | - | - | - | - | - | + | - | - | + | + | - |
| EBLV-2[b] | + | - | + | - | + | - | - | + | + | + | - | - | + | - | - | + | + | + | - | + | - |
| Aravan virus | - | - | + | - | + | + | - | + | + | + | - | - | - | - | - | + | - | + | - | + | - |
| Khujand virus | o | - | - | - | + | + | - | - | - | - | - | - | - | - | - | + | - | + | + | + | - |
| Rabies, Red fox (West Europe)[b] | + | + | + | + | + | + | - | + | + | + | + | - | + | + | + | + | - | + | + | + | - |
| Rabies, Red fox (Caucasus) | + | + | + | + | + | + | - | + | + | + | + | o | - | + | + | + | - | + | + | + | - |
| Rabies, CVS | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - |

[a]N-MAbs, antinucleocapsid monoclonal antibodies; –, absence of reaction; zero, reduced reaction with $10^x$ less diluted antibody; +, positive reaction; WCBV, West Caucasian bat virus; .EBLV, European bat lyssavirus; CVS, challenge virus standard.
[b]Patterns obtained from Smith (11).

entire N and other genes should be conducted to refine the phylogenetic relationships of both these viruses.

## Conclusions

Estimating the potential public health significance of these two newly recognized lyssaviruses is critical. Other bat lyssaviruses cause fatal human encephalitis, even in so-called "rabies-free" countries (2,3,13). Given bat mobility and the opportunity for infecting new areas quickly, no major geographic area can be considered truly free from lyssaviruses. For example, the Irkutsk Province was considered free of rabies for 35 years before the Irkut virus was isolated. Additionally, although the Caucasus had been considered as a rabies-endemic area, virus reservoirs were identified only among the canids. Public health authorities need to be aware of the potential of bats to transmit lyssaviruses and increase surveillance and public education. Attention should focus on the protective efficacy of commercially available rabies virus vaccines and immune globulins against these novel nonrabies lyssaviruses, before human infection occurs.

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Dr. Botvinkin is the deputy director of Plague Control Research Institute of Siberia and the Far East in Irkutsk, Russia. Since 1975, his research interests have included rabies and rabies-related viruses in the former Soviet Union.

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Address for correspondence: Ivan V. Kuzmin, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS G33, Atlanta, GA 30333, USA; fax: 404-639-1564; email: ibk3@cdc.gov

# Human Metapneumovirus and Respiratory Syncytial Virus, Brazil

**Luis E. Cuevas,* Abubaker M. Ben Nasser,* Winifred Dove,† Ricardo Q. Gurgel,‡ Julie Greensill,* and C. Anthony Hart†**

We describe the epidemiologic and clinical characteristics of 111 children attending clinics and hospitals in Aracaju, northeast Brazil, with acute respiratory infections attributable to human metapneumovirus (HMPV), respiratory syncytial virus (RSV), or both in May and June 2002. Fifty-three (48%) children were infected with RSV alone, 19 (17%) with HMPV alone, and 8 (7%) had RSV/HMPV co-infections.

Human metapneumovirus (HMPV) was first identified in the Netherlands in 2001 (1) and was implicated as a potential etiologic agent for respiratory infections. Since then, the virus has been reported from other European countries (2–6), Asia (7–9), and North America (10–13), findings that suggest it has a worldwide distribution. However, HMPV has not been reported from South America. We describe the epidemiologic and clinical characteristics of 111 children attending clinics and hospitals in Aracaju, northeast Brazil, with acute respiratory infections attributable to HMPV, respiratory syncytial virus (RSV), or both.

## The Study

Children <3 years of age attending two health centers, one public reference hospital (Joao Alves), and a private hospital in Aracaju were invited to participate in our study during April and May 2002. These months correspond to the beginning of the rainy season, when most cases of bronchiolitis occur. The health centers provide 24-hour medical services for acute illnesses; patients with severe health problems are referred to the public hospital. The small private hospital caters to self-referred and transferred patients (mostly from the public hospital). Children with diagnoses of acute lower respiratory infections (ALRI) were recruited consecutively after informed parental consent. The diagnosis of ALRI was based on the presence of cough, tachypnea, chest indrawing, or wheeze of <7 days' duration and followed the World Health Organization's standard protocol for research on ALRI (14).

Nasopharyngeal secretions were collected by using sterile mucous traps (Maesk Medical A/S, Bettina Bay, Denmark). Immediately after collection, aspirates were mixed with phosphate-buffered saline, transferred into 2-mL cryotubes, and kept frozen at –80°C until analyzed. Hypoxia levels were measured by pulse oximetry before the use of oxygen, and children were treated according to local guidelines for the management of ALRI.

Detection of the HMPV genome was performed by reverse transcription–polymerase chain reaction amplification (RT-PCR) of the matrix (M), fusion (F) and nucleocapsid (N) protein genes, as described previously (5). Selected PCR products were cloned into a TA cloning vector (pGEM-T, Promega, UK), and the sequence was determined to confirm the identity of the virus detected by the PCR reaction. Samples were defined as HMPV positive if at least two of the RT-PCR test results were positive, although in practice all positive samples had at least two positive PCRs. Detection of RSV by RT-PCR was as described by Fletcher et al. (15).

A total of 111 children (57 from the health centers, 25 from Joao Alvez Hospital, and 29 from the private hospital) were recruited. Their ages ranged from 1 to 30 months (median 7 months). Fifty-three (48%) children were infected with RSV alone, 19 (17%) with HMPV alone, and 8 (7%) with both (co-infections). Forty-six (88%) of the RSV cases were of group A and 6 (12%) group B.

The incidence of ALRI increased during the 10 weeks of the study from no cases in the first 2 weeks, 4 cases in the next 2 weeks, and up to 25 cases in week 9 (Figure 1). RSV and HMPV infections appeared to coincide in time during the period of the study.

The characteristics of the children are described in the Table. The incidence of HMPV and RSV varied according to the enrollment site. Eighteen (32%) of the 57 patients attending the health centers had HMPV compared to 1 (4%) of the 25 infants attending the reference hospital ($p = 0.01$). In contrast, RSV was significantly ($p = 0.01$) more frequently detected in patients attending the referral hospital (17 [68%] of 25) than the health centers (23 [40%] of 57). The age distribution of the infected infants is shown in Figure 2. The greatest number of cases of RSV was observed in children ≤12 months of age. HMPV infection appeared to be more frequent in children 6–24 months of age, although this finding was not statistically significant.

All children had coughing; 61 (55%) were wheezing, which was audible without auscultation; and 34 (31%) had chest indrawing. The mean respiratory rate on consultation

*Liverpool School of Tropical Medicine, Liverpool, United Kingdom; †University of Liverpool, Liverpool, United Kingdom; and †Federal University of Sergipe, Aracaju, Brazil

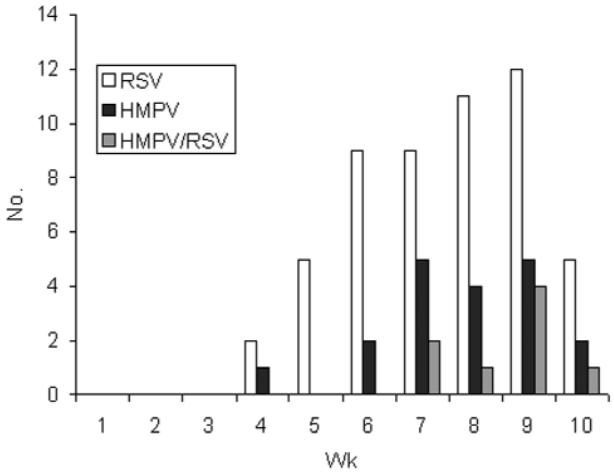


Figure 1. Number of children with respiratory syncytial virus (RSV), human metapneumovirus (HMPV), and RSV/HMPV co-infection by study week (week 10 incomplete).

was higher in patients with RSV than in patients with HMPV infection (p = 0.03). Similarly, wheezing was more often audible in children infected with RSV (59%) than in children with HMPV (47%) or RSV/HMPV co-infections (25%), although this finding did not reach statistical significance (chi square for trend, p = 0.07). Eighteen (16%) children had severe hypoxia with $pO_2$ <90%, and 44 (40%) had $pO_2$ <94%. The presence of the viruses was associated with the degree of hypoxia. Five (26%) of the 19 children with HMPV had $pO_2$ <94% compared to 25 (47%) of the 53 with RSV and 5 (63%) of the 8 with RSV/HMPV co-infection (chi square for trend, p = 0.05). Severe hypoxia ($pO_2$ <90%), however, was less frequent in children with HMPV (1 [5%] of 19) or RSV/HMPV co-infections (1 [13%] of 8) than in children with RSV infection (14 [26%] of 53 (chi square for trend, p = 0.04). The lower prevalence of hypoxia suggests that children with HMPV had milder illnesses, since only 5 (26%) of the 19 HMPV patients and 2 (25%) of the 8 patients with RSV/HMPV co-infections were admitted to hospital compared with 27 (51%) of the 53 with RSV infection, although this finding was not statistically significant.

## Conclusions

This study is the first to describe HMPV in Latin America. To date, information on the clinical signs and symptoms and epidemiology of HMPV infection is limited. Our study indicates that HMPV and RSV may have similar signs and symptoms and can present as a co-infection.

The incidence of HMPV increased at the same time that the number of cases with RSV was increasing. HMPV, however, was more prevalent in children attending peripheral clinics than the referral hospital. Similarly, children with HMPV were less likely to be hypoxic and had lower respiratory rates than those with RSV, which suggests that HMPV infection may result in milder clinical signs and symptoms. Further studies, however, are necessary to explore if coinfected children are more or less likely to have a worse clinical outcome than children infected with only one virus. A recent report of hospitalized children in intensive care suggested that dual infections could be asso-

Table. Characteristics of children with human metapneumovirus (HMPV), respiratory syncytial virus (RSV), and RSV/HMPV co-infections[a]

| Characteristics | HMPV n = 19 | RSV n = 53 | RSV/HMPV co-infection n = 8 | HMPV/RSV negative n = 31 |
|---|---|---|---|---|
| Age in mo, mean (SD) | 10.1 (5.4) | 8.9 (7.2) | 8.2 (6.0) | 9.8 (7.7) |
| Recruitment site[b] | | | | |
| Health center | 12 (21) | 23 (40.3) | 6 (10.5) | 16 (28.1) |
| Private hospital | 6 (20.7) | 13 (44.8) | 2 (6.9) | 8 (27.6) |
| Public hospital | 1 (4) | 17 (68) | 0 (0) | 7 (28) |
| Male:female (% male) | 12:7 (63) | 36:17 (68) | 4:4 (50) | 21:10 (68) |
| Birthweight <2,500 g | 4 (21) | 2 (4) | 0 (0) | 2 (6.5) |
| Previous admission | 2 (10) | 7 (13) | 3 (38) | 1 (3) |
| History of allergies | 2 (11) | 6 (11) | 1 (13) | 4 (13) |
| Clinical signs and symptoms | | | | |
| Respiratory rate, mean (SD) | 44 (10.1) | 51 (12.3)[c] | 54 (16.2) | 46 (10.1) |
| Temperature >37.5°C | 15 (79) | 43 (81) | 8 (100) | 23 (74) |
| Runny nose | 18 (95) | 45 (85) | 6 (75) | 26 (84) |
| Cyanosis | 0 (0) | 4 (7.5) | 0 (0) | 2 (6.5) |
| Cough | 19 (100) | 53 (100) | 8 (100) | 31 (100) |
| Wheezing | 9 (47.4) | 31 (58.5) | 2 (25%) | 19 (61.3) |
| Chest indrawings | 4 (21) | 19 (36) | 3 (37.5) | 8 (25.8) |
| $pO_2$ <90% | 1 (5.3) | 10 (18.9) | 0 (0) | 1 (3.2) |
| $pO_2$ <94%[c] | 5 (26.3) | 25 (47.2) | 5 (62.5) | 9 (29) |

[a]Figures are frequencies (%), unless otherwise specified.
[b]Health centers vs. public hospital, p < 0.01. Percentages are row percentages.
[c]p < 0.05.

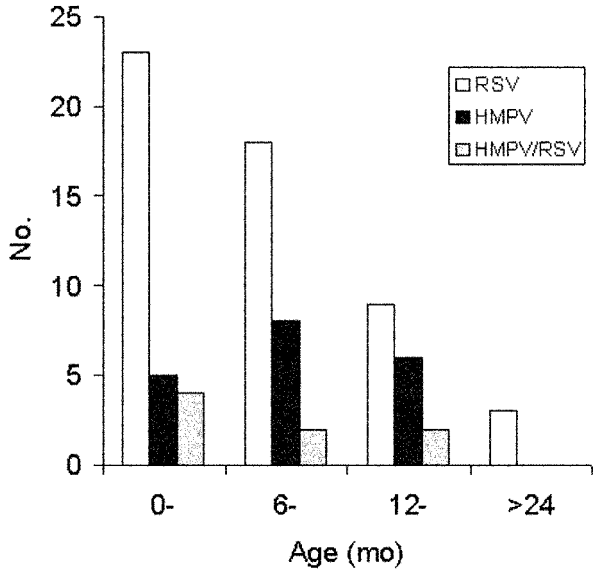


Figure 2. Number of children with respiratory syncytial virus (RSV), human metapneumovirus (HMPV), and RSV/HMPV co-infection by age.

ciated with increased severity (5). An alternative explanation to reconcile these findings is the possibility that different HMPV subgroups (2,9,10,13,16) produce clinical syndromes of varying severity.

Caution is necessary to interpret our findings as this was a cross-sectional study. Virus in nasopharyngeal secretions does not prove that it is the cause of the respiratory symptoms, as we did not investigate the prevalence of the virus in asymptomatic children or the natural history of the infection. HMPV, however, has been associated with community-acquired respiratory illnesses (2,6,9–12) and severe ALRI in immunocompromised patients (17,18), and the virus was likely responsible for the clinical illness in our children. Several studies have also described an association between HMPV and acute wheezing (3–5,8). Although wheezing was a feature in 47% of the HMPV-infected children, a greater proportion (59%) of children infected with RSV had wheezing. The association between RSV infection and asthma is well studied. However, most children are exposed to RSV infections before the age of 2 years, and a combination of factors is likely required (19). The coincidental timing of RSV and HMPV might have led us to miss the true cause. The role of HMPV in ALRI and asthma merits further investigation. Future studies should aim to establish the natural history and clinical outcome of these infections.

Dr. Cuevas is a senior lecturer in epidemiology at the Liverpool School of Tropical Medicine. His main research interest is investigating the epidemiology of meningitis, tuberculosis, acute respiratory infections, and diarrhea.

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Address for correspondence: Luis E. Cuevas, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK; fax: 0044 151 705368; email: lcuevas@liv.ac.uk

# *Actinomyces odontolyticus* Bacteremia

**Lawrence A. Cone,*† Millie M. Leung,† and Joel Hirschberg*†**

We describe two immunosuppressed female patients with fever and *Actinomyces odontolyticus* bacteremia, a combination documented once previously in an immunocompetent male patient. The patients were treated with doxycycline and clindamycin; these drugs, with β-lactams, are effective treatment for *A. odontolyticus* infections.

Actinomycosis is a disease of antiquity, having most likely infected the jaw of a fossil rhinoceros (1) and the ribs of a man discovered in southeastern Ontario, Canada, who by radiocarbon dating lived 230 A.D. ± 55 (2). In 1877, Bollinger and Harz (3) named the genus *Actinomyces* when they described the etiologic agent of bovine actinomycosis ("lumpy jaw") and called it *Actinomyces bovis*. However, this organism has never been convincingly proven to cause actinomycosis in humans (4), nor has it ever been isolated from human mucosa or other human sources.

The major human pathogen for actinomycosis, *A. israelii,* was identified in two patients in 1878 and fully delineated by Israel (5). In 1891, Wolff and Israel (6) described the cultural characteristics and its anaerobic growth. Since then, studies have identified *A. naeslundii, A. viscosus, A. pyogenes, A denticolens, A. howellii, A. hordeovulneris,* and *A. meyeri* in humans as well as in dogs and cats. Actinomycosis is the most common infectious disease of kangaroos (7).

In 1958, Batty (8) isolated *A. odontolyticus* from persons with advanced dental caries. During the ensuing 40+ years, 23 patients with invasive infection caused by *A. odontolyticus* have been described in North America, Europe, and Asia (9–25). Thirteen patients had pulmonary, cardiopulmonary or mediastinal disease, 4 had soft tissue infections, 2 had abdominal involvement, 2 had pelvic involvement, 1 had a brain abscess, and 1 other had bimicrobial bacteremia with *Fusobacterium necrophorum*. We describe two cases, in 1998 and 1999, involving immunocompromised patients with fever and bacteremia resulting from *A. odontolyticus* and consider the 23 previously described.

*Eisenhower Medical Center, Rancho Mirage, California, USA; and †Harbor-University of California at Los Angeles Medical Center, Torrance, California, USA

## Case Reports

### Patient 1

In March 1999, a 62-year-old white woman who had worked as a chemotherapy nurse from 1973 to 1979 sought treatment at Eisenhower Medical Center after having pain in her left knee for 2 weeks. Magnetic resonance imaging indicated a left lateral meniscus tear. A routine preoperative complete blood count (CBC) showed a leukocyte count of 6.8 x $10^9$/L, hemoglobin (Hb) of 82 g/L, hematocrit (Hct) of 0.26, and a thrombocyte count of 95 x $10^9$/L. Examination of the peripheral smear demonstrated frequent blasts with no discernible Auer rods. Flow cytometric analysis of a bone marrow biopsied sample showed involvement with > 30% blasts that were positive for CD13, CD33, CD34, CD117, CD19, and TdT-negative. The markers and morphologic characteristics were consistent with acute myelocytic leukemia, monocytes with aberrant expression of CD19, a B-cell marker. Cytogenetics showed a normal 46,XX female chromosome complement. Fluorescence in situ hybridization (FISH) using polymerase chain reaction (PCR) techniques showed no evidence for monosomy, trisomy 8, or partial deletions of the long arm of chromosome 5 or 7.

Induction chemotherapy consisting of 3 days of idarubicin at 12 mg/m² daily and 7 days of cytosine arabinoside by continuous infusion at 100 mg/m² was given to the patient. Four days post-treatment, a temperature of 39°C developed in the patient. The CBC showed the leukocyte count was 6.8 x $10^9$/L, Hb was 82 g/L, Hct was 0.26, and thrombocyte count was 93 x $10^9$/L. Two of four blood cultures (using blood agar, CNA, and Brucella agar) grew *A. odontolyticus* in 24–48 hours. Because of a penicillin allergy, 100 mg of doxycycline was given intravenously to the patient every 12 hours for 2 weeks. Follow-up blood cultures were sterile. The patient's dental health appeared normal and no source for the bacteremia was identified. The patient entered complete remission. The second cycle of consolidation chemotherapy was also complicated by fever. *Capnocytophaga* spp was isolated from the patient's blood using blood agar supplemented with $CO_2$. A fastidious streptococcus that did not grow on agar was also isolated. Oral surgical consultation was obtained and evidence for a dental abscess was uncovered. The abscess was treated with clindamycin. Thirty months after the first consolidation chemotherapy, the patient remained in remission.

### Patient 2

A 69-year-old white woman had experienced good health until she sought treatment in May 1998 at Eisenhower Medical Center. She reported a 6-month history of worsening generalized abdominal pain, nausea, vomiting, diarrhea, and weight loss. Blood serologic tests indi-

cated an erythrocyte sedimentation rate (ESR) of 62 mm/h and positive antinuclear antibodies (ANA) at a titer of 640 (homogeneous) but negative cryoglobulins, lupus anticoagulant, antineutrophil cytoplasmic antibodies (c-ANCA), and cardiolipin antibodies. Quantitative immunoglobulins were normal; an upper gastrointestinal series and computerized tomographic scan of the abdomen showed no abnormalities. A colonoscopy showed diverticulosis coli with no other deformities. Magnetic resonance angiography showed substantial stenosis of the right subclavian, right brachial, superior mesenteric, bilateral renal, and external iliac arteries. Giant cell arteritis was diagnosed in the absence of a confirming biopsy, and the patient received 60 mg prednisone daily. The patient showed no measurable clinical improvement for 7 days. Consequently, azathioprine therapy at 50 mg daily was initiated. Four days later, a temperature of 39°C and chills developed in the patient. Blood cultures using blood agar, CNA, and Brucella agar grew *A. odontolyticus* in 24–48 hours. Because of allergies to penicillin, cephalosporin, and tetracycline, clindamycin was given to the patient for 14 days. The recovery was uneventful, and clinical evidence did not indicate dental disease.

*Actinomyces odontolyticus* is an anaerobic, facultative capnophilic, gram-positive, nonsporulating, non–acid fast, non-motile, irregularly staining bacterium. Sometimes short or medium-sized rods resembling diphtheroids are seen. Shorter rods resembling propionibacteria are frequently seen with *A. odontolyticus* and may be arranged in palisades as well as other diphtheroidal arrangements. On blood agar, the bacteria develop as small, irregular, whitish colonies that are smooth to slightly granular and show a dark red pigment when mature (2–14 days). This pigmentation is most obvious when the cultures are left standing in air at room temperature after primary anaerobic isolation. The organism also grows well on CNA and Brucella agar.

Definitive identification is made by negative catalase and oxidase tests, the reduction of nitrate to nitrite, filamentation of microcolonies, and absence of growth at pH 5.5. Generally, the fermentation reactions are variable.

*A. odontolyticus* was speciated in these two case-patients by using the RapID ANA II System (Remel Inc., Lenexa, KS), a qualitative microsystem using conventional and chromogenic substrates for the identification by disc diffusion of anaerobic bacteria of human origin. Both strains were sensitive to penicillin, ampicillin, cephalosporins, tetracycline, clindamycin, chloramphenicol, and erythromycin.

## Discussion

The previously described and the two present case-patients are summarized in the Table. Most are men (14 vs. 9 women, with 2 of unknown sex), and the mean age is 50 years. Five patients were immunosuppressed: two had received prednisone, one had received chemotherapy, and two had organ transplants. Two of the 25 patients were known to be alcoholic, and 3 were noted to have periodontal disease.

Clinical disease in patients with *A. odontolyticus* closely resembles disease caused by *A. israelii* and other actinomyces species. Similar to *A. israelii* infections, those caused by *A. odontolyticus* primarily involve the cervicofacial regions, the chest, abdomen, and pelvis with rare involvement of the central nervous system, bones, and joints. Additional similarities include a more frequent occurrence in men than women and a peak incidence in the middle decades of life. Clinical features in 97% of 181 patients with actinomycosis including the following: mass or swelling, pulmonary disease, draining abscesses, abdominal disease, dental disease, and intracranial infection (26).

Only two deaths were recorded: one patient died with a brain abscess and another with mediastinitis. The patients responded to various β-lactam therapies including penicillins, cephalosporins, carbapenems as well as macrolides, lincosides, and tetracycline. Responses to imidazoles were unpredictable, and the patient with a brain abscess caused by *A. odontolyticus* was administered metronidazole and did not recover (11).

## Conclusions

As with all other actinomycotic diseases, *A. odontolyticus* is an endogenous infection arising from the mucous membranes. Batty (8), after some experience, was able to isolate the organism from the dentine of 90% of subjects studied, while Mitchell and Crow (27) isolated *A. odontolyticus* in female genital tract specimens from 4.8% of women fitted with intrauterine contraceptive devices, in 4% of women with pelvic inflammatory disease, and in 1.8% of women without pelvic inflammatory disease.

The capacity of actinomycetes to colonize mucosal surfaces and dentine appears to depend on two distinct fimbriae, type 1 and type 2, that bind preferentially to salivary acidic proline-rich proteins and to statherin, or to β-linked galactose or galactosamine structures on epithelial or bacterial surfaces, respectively (28).

We believe that patient 1 (with acute leukemia) had a dental abscess, probably secondary to *A. odontolyticus,* that served as a portal for the bacteremia. Of the 23 previously reported case-patients of *A. odontolyticus* infection, only one (an otherwise healthy 20-year-old man [9]) had bacteremia. The two reported case-patients were women: one had received chemotherapy for acute granulocytic leukemia and the other had received high dose corticosteroids for vasculitis. Immunosuppression probably played a major role in the etiology of bacteremic *A. odontolyticus*

Table. Reported cases of *Actinomyces odontolyticus* infection

| Case | Y (Ref) | Disease | Age/Sex | Underlying disease | Presentation | Treatment |
|---|---|---|---|---|---|---|
| 1 | (PR) | Bacteremia | 62/F | Acute myelogenous leukemia | Fever | Doxycycline |
| 2 | (PR) | Bacteremia | 69/F | Vasculitis, immunosuppression | Fever, chills | Clindamycin |
| 3 | 1999 (25) | Pericardial, pleural effusions | 68/M | S/P resection malignant gastric polyp | Fever, dyspnea | Cefriaxone, amoxicillin |
| 4 | 1997 (24) | Empyema | 50/M | S/P pneumonectomy for tuberculosis and aspergilloma, alcoholism | Fever, dyspnea, chest pain | N/S |
| 5 | 1997 (23) | Mediastinitis | 43/M | Heart-lung transplant, immunosuppression | Sternal wound drainage | Penicillin |
| 6 | 1996 (23) | Pneumonia | 61/M | Lung transplant, immunosuppression | Chest pain | Penicillin |
| 7 | 1995 (22) | Empyema | 40/M | Chronic bronchitis, alcoholism | Fever, chest pain, cough | Penicillin |
| 8 | 1994 (21) | Pneumonia, cutaneous abscess | 52/M | Periodontal disease, alcoholism | Fever, weight loss, cutaneous drainage | Penicillin |
| 9 | 1993 (20) | Thoracic abscess | N/S | N/S | N/S | N/S |
| 10 | 1992 (19) | Pneumonia | 52/F | Bronchiectasis | Fever, weight loss | Imipenem, tetracycline |
| 11 | 1992 (18) | Empyema | 38/F | Periodontal disease | Fever, chest pain, dyspnea, cough, weight loss | Penicillin |
| 12 | 1990 (17) | Pleural lesion, chest wall erosion, spinal and muscle abscesses | 58/F | None | Fever, chest pain, weight loss | Penicillin, metronidazole |
| 13 | 1985 (16) | Submaxillary gland | 65/M | None | Swelling of neck, lymphadenopathy | Tetracycline |
| 14 | 1985 (16) | Arm abscess | 47/M | None | Fever, swelling, erythema of arm | Penicillin, gentamicin, ornidazole |
| 15 | 1985 (16) | Pelvic infection | 30/F | None | Infected intrauterine device | Device removed, metronidazole |
| 16 | 1985 (16) | Pelvic abscess | 54/F | Alcoholism | Fever, pelvic pain | Tobramycin |
| 17 | 1985 (16) | Thumb abscess | 40/M | None | Fishbone injury to thumb | Cephalothin |
| 18 | 1985 (16) | Bacteremia | 19/M | None | Confusion, icterus, fever | Penicillin, ornidazole |
| 19 | 1985 (15) | Enterocutaneous fistula | 78/M | Diverticulitis | Fecal fistula, abdominal abscess | Erythromycin |
| 20 | 1982 (14) | Cholestasis | 43/F | None | Abdominal pain | Doxycycline |
| 21 | 1979 (13) | Pulmonary abscess | 61/F | Rheumatoid arthritis, prednisone | Fever, dyspnea, chest pain | Tetracycline, clindamycin |
| 22 | 1979 (12) | Brain abscess | 34/M | None | Headache, vomiting, fever | Penicillin, metronidazole |
| 23 | 1977 (11) | Empyema | N/S | N/S | N/S | N/S |
| 24 | 1977 (10) | Cellulitis | 54/M | None | Cheek mass | Penicillin |
| 25 | 1974 (9) | Thoracic wall abscess | 26/M | None | Subcutaneous chest mass | Clindamycin, penicillin |

[a](PR), present report; F, woman; M, man; S/P, status post; N/S, not stated.

infection. Further studies to evaluate possible mechanisms would be appropriate.

Dr. Cone is an infectious diseases clinician at the Eisenhower Medical Center, assistant clinical professor of medicine at University of California at Los Angeles, and clinical professor of medicine at University of California, Riverside. His research interests include genetics, immune deficiencies, and sepsis.

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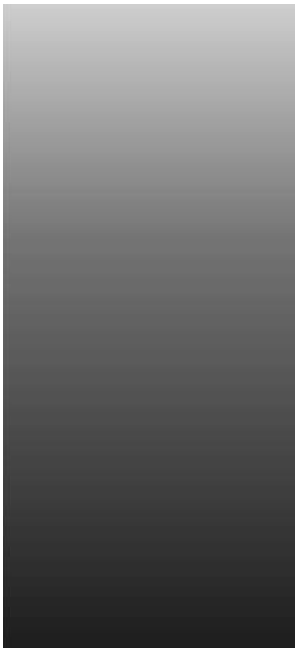
Address for correspondence: Lawrence A. Cone, Eisenhower Medical Center, Probst Professional Building, Suite #308, 39000 Bob Hope Drive, Rancho Mirage, CA 92270 USA; fax: 760 773-3976; email: laconemedico@aol.com





# *Mycobacterium tuberculosis* Beijing Genotype and Risk for Treatment Failure and Relapse, Vietnam

**Nguyen Thi Ngoc Lan,* Hoang Thi Kim Lien,* Le B. Tung,* Martien W. Borgdorff,†‡ Kristin Kremer§, and Dick van Soolingen§**

Among 2,901 new smear-positive tuberculosis cases in Ho Chi Minh City, Vietnam, 40 cases of treatment failure and 39 relapsing cases were diagnosed. All initial and follow-up *Mycobacterium tuberculosis* isolates of these case-patients had (nearly) identical restriction fragment length polymorphism patterns, and the Beijing genotype was a significant risk factor for treatment failure and relapse (odds ratio 2.8, 95% confidence interval 1.5 to 5.2).

The Beijing genotype is widespread in Asia (1–3), and has been involved in outbreaks of multidrug-resistant tuberculosis in various parts of the world, including Cuba, Germany, Russia, and Estonia (4–7). The W strain, which caused a large outbreak of multidrug-resistant tuberculosis in the United States, is a variant of the Beijing genotype (8–10). The Beijing genotype is emerging in Vietnam in association with drug resistance in this region (11).

In a recent study on acquired drug resistance in Ho Chi Minh City, Vietnam, drug resistance at time of enrollment in the study was shown to be an important risk factor for treatment failure and for relapse of tuberculosis after treatment was completed successfully (12). We used the materials collected for this study to determine the extent to which the Beijing genotype is a risk factor for treatment failure or relapse.

The methods of this study have been described previously (12). In brief, 2,901 new case-patients with smear results positive for *Mycobacterium tuberculosis* were enrolled in Ho Chi Minh City, Vietnam, from August 1996 through July 1998. After a case was diagnosed at the district tuberculosis center, a sputum sample from the case-patient was sent to the reference laboratory, for a repeat microscopy examination of the sputum smear to confirm the diagnosis and to be stored at –20°C. All patients received the standard regimen of the National Tuberculosis Program, i.e., 2 months of streptomycin, isoniazid, rifampicin, and pyrazinamide, followed by 6 months of isoniazid and ethambutol (2SHRZ/6HE). When treatment failure (defined as a positive sputum smear 5 or 8 months after the onset of treatment) or relapse (defined as a positive sputum smear within 2 years after scheduled treatment cessation) was noted, another sputum sample was collected, and both samples were cultured and tested for drug susceptibility with the proportion method. Restriction fragment length polymorphism (RFLP) typing was performed by using insertion element IS*6110* as a probe (13,14) to exclude reinfection and laboratory cross-contamination.

A random sample of sputum samples was collected at enrollment for culture and sensitivity testing from 10% of patients who had not experienced treatment failure or relapse (controls). This sample size would allow approximately two controls per case-patient. We performed spoligotyping on the sputum samples of case-patients who had experienced treatment failure or relapse and controls to identify the samples that belonged to the Beijing genotype (15). The Beijing genotype was defined as strains without spacers 1–34 and the presence of (at least 3) the spacers 35–43 (16).

Over the enrollment period, 6,113 new smear-positive tuberculosis patients began a treatment regimen, 2,901 of whom were included in the study. Slightly more men were enrolled than women (age-adjusted odds ratio [OR] 1.2, 95% confidence interval [CI] 1.0 to 1.3), and enrollment was particularly low in those ≥65 years of age (sex-adjusted OR 0.3, 95% CI 0.2 to 0.4). Of the 2,901 enrolled patients, 2,568 (88%) recovered, and 12 (0.4%) completed treatment; in 125 (4.3%), treatment failed; 63 (2.2%) died; 53 (1.8%) were transferred out; and 80 (2.8%) did not complete the study. Through December 1999, a total of 168 case-patients who experienced a relapse (6.5% of those cured or with treatment completed) were identified. Forty of 125 case-patients whose treatment failed and 39 of 168 case-patients who had a relapse had two positive cultures with nearly identical RFLP patterns (12). Spoligotyping results were available for 136 controls.

Case-patients were somewhat less likely than controls to be female and tended to be somewhat older than controls. However, these differences were not significant. Primary drug resistance (in comparison with full susceptibility) was a strong risk factor for treatment failure or relapse with combined ORs of 3.4 for streptomycin monoresistance, 4.2 for isoniazid monoresistance, and 23

*Pham Ngoc Thach Tuberculosis and Lung Diseases Centre, Ho Chi Minh City, Vietnam; †Royal Netherlands Tuberculosis Association, The Hague, the Netherlands; ‡University of Amsterdam, the Netherlands; and §National Institute of Public Health and the Environment, Bilthoven, the Netherlands

for other susceptibility patterns (Table). The Beijing genotype was associated with treatment failure (OR 3.3 95% CI 1.3 to 8.3; $p < 0.01$) and relapse (OR 2.4 95% CI 1.0 to 5.7; $p < 0.05$). In view of the small numbers and similar odds ratios, these two groups were combined (OR 2.8, 95% CI 1.5 to 5.2) (Table). The association between the Beijing genotype and treatment failure or relapse hardly changed when taking into account primary drug resistance, age, and sex (OR 3.2, 95% CI 1.4 to 7.1). We conclude that the Beijing genotype is a risk factor for treatment failure and relapse in Vietnam, irrespective of primary drug resistance. This finding suggests that infections with Beijing genotype strains are more persistent than infections with other *M. tuberculosis* strains, which may explain the emergence of Beijing genotype strains in this region (11).

This study had limited power to detect risk factors for relapse and treatment failure, mainly because of the relatively small numbers of case-patients in those categories. Recruiting a larger number of controls could not change this, since the selection of more than two controls per case, while increasing workload, has relatively little impact on the statistical power of the analysis. However, since the association between the Beijing genotype and treatment failure or relapse was strong, the association was significant despite limited power.

Beijing genotype strains may have several selective advantages over other genotypes of *M. tuberculosis*. In many, but not all, areas where Beijing genotype strains are prevalent, this genotype is associated with resistance to antituberculosis drugs (17). The basis for this correlation has so far not been disclosed. However, recent findings indicated that exclusively in Beijing genotype strains, mutations are present in putative mutator genes (18). This finding may indicate that Beijing genotype strains have a higher ability than other strains to allow particular critical mutations in resistance genes, which enables them to acquire resistance to the drugs used in a standard treatment regimen.

This enhanced flexibility due to alterations in the DNA repair mechanism of Beijing genotype bacteria may also play a role in the interaction with the host immune defense system to deal with the less favorable conditions like exposure to oxygen and nitrogen radicals in intracellular environment. Extended research on the immunopathology caused by *M. tuberculosis* strains of different genotypes in a BALB/c mouse model has shown that most, but not all, Beijing genotype strains cause a more severe pathology, but a reduced immune response in comparison to other genotypes of *M. tuberculosis* (19).

If Beijing genotype strains have a selective advantage over other genotypes of *M. tuberculosis,* this may have important implications for future tuberculosis control. The enhanced capability to develop resistance and to interact with the host immune defense system may facilitate the spread of tuberculosis in Asia and in other areas. Currently, a worldwide survey is being conducted to measure the global spread of this genetically conserved group of *M. tuberculosis* strains and its association with resistance, active transmission (young age), and other factors. Although the conservation of Beijing genotype strains in Asia is highly pronounced, the conserved population structure of *M. tuberculosis* in other high-prevalence areas such

Table. Characteristics at enrollment of case-patients who experienced treatment failure and relapse and of controls who did not experience treatment failure, relapse, or die[a]

| | | | | | Crude | | Adjusted[b] | | Adjusted[c] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Characteristic | Failure | Relapse | All case-patients | Controls | OR | 95% CI | OR | 95% CI | OR | 95% CI |
| Sex | | | | | | | | | | |
| Male | 28 | 36 | 64 | 104 | 1 | | | | 1 | |
| Female | 12 | 3 | 15 | 39 | 0.63 | 0.32 to 1.22 | | | 0.55 | 0.22 to 1.38 |
| Age group (y) | | | | | | | | | | |
| 15–34 | 15 | 9 | 24 | 60 | 1 | | | | 1 | |
| 35–54 | 22 | 25 | 47 | 72 | 1.63 | 0.90 to 3.0 | | | 2.0 | 0.90 to 4.6 |
| ≥55 | 3 | 5 | 8 | 11 | 1.82 | 0.65 to 5.1 | | | 2.4 | 0.60 to 9.9 |
| Genotype | | | | | | | | | | |
| Beijing | 32 | 29 | 61 | 75 | 2.8 | 1.48 to 5.15 | 2.5 | 1.2 to 5.2 | 3.2 | 1.4 to 7.1 |
| Other | 8 | 10 | 18 | 61 | 1 | | 1 | | 1 | |
| Unknown | | | | 7 | | | | | | |
| Resistance pattern | | | | | | | | | | |
| Fully susc. | 4 | 13 | 17 | 101 | 1 | | 1 | | 1 | |
| S only | 3 | 9 | 12 | 21 | 3.4 | 1.41 to 8.2 | 4.0 | 1.6 to 9.9 | 3.9 | 1.54 to 9.9 |
| H only | 3 | 4 | 7 | 10 | 4.2 | 1.39 to 12 | 4.7 | 1.5 to 15 | 5.0 | 1.54 to 16 |
| Other | 30[d] | 13[e] | 43 | 11 | 23 | 10.0 to 54 | 23 | 9.7 to 55 | 26 | 10.3 to 64 |
| TOTAL | 40 | 39 | 79 | 143 | | | | | | |

[a] OR, odds ratio; CI, confidence interval; susc., susceptible.
[b] Adjusted for genotype and resistance pattern.
[c] Adjusted for age, sex, genotype, and resistance pattern.
[d] Of these 30, 12 had resistance to H and S, 1 to H, S, and E, 10 to H, R, and S, and 7 to H, R, S, E.
[e] Of these 13, 11 had resistance to H and S, and 2 to H, S, and E.

as Africa also merits further research on the possible development of selective advantages.

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Dr. Lan is the director of the National Tuberculous Rreference Laboratory in Ho Chi Minh City, Vietnam, and is responsible for science and technology at the Pham Ngoc Thach Hospital (Tuberculosis and Lung Diseases Hospital for the South of Vietnam). Her research interests include the microbiology, epidemiology, and molecular biology of tuberculous.

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Address for correspondence: Dick van Soolingen, National Institute of Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, the Netherlands; fax: +31 30 2744418; email: d.van.soolingen@rivm.nl

# *Baylisascaris procyonis* in the Metropolitan Atlanta Area

**Mark L. Eberhard,* Eva K. Nace,* Kimberly Y. Won,* George A. Punkosdy,* Henry S. Bishop,* and Stephanie P. Johnston***

*Baylisascaris procyonis*, the raccoon roundworm responsible for fatal larva migrans in humans, has long been thought to be absent from many regions in the southeastern United States. During spring 2002, 11 (22%) of 50 raccoons trapped in DeKalb County, Georgia, had *B. procyonis* infection. The increasing number of cases highlight this emerging zoonotic infection.

*Baylisascaris procyonis* was first described from specimens recovered from raccoons in Europe (1), although the first report of raccoon infections with the worm came from New York in 1933 (2). The first recognized human case was reported in 1984 in a 10-month-old child in Pennsylvania (3). Since then, at least 11 additional cases of severe or fatal *B. procyonis* encephalitis have been identified in Illinois, California, Michigan, Minnesota, New York, and Oregon (4).

The distribution of *B. procyonis* in the United States has been well recorded in some areas and poorly documented in other areas. The infection appears to be common in the Midwest, Northeast, and Middle Atlantic regions (5). Recently, *B. procyonis* has been found to be common in parts of California (6–8). However, *B. procyonis* historically has not been reported in the southeastern United States, except in mountainous areas. There is one anecdotal account of the infection in raccoons in central Georgia (9), and the literature cites an unpublished report of 1 out of 110 animals in north Georgia being infected (10), but no substantiated reports of the infection in Georgia have been found.

As part of ongoing studies that use animal dracunculiasis (*Dracunculus insignis*) as a model for the study of human dracunculiasis, raccoons were examined for preemergent female *D. insignis* worms. This study provided an opportunity to examine the animals for *B. procyonis*. We describe the occurrence of *B. procyonis* in the metropolitan Atlanta area (DeKalb County, Georgia).

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

## The Study

Raccoons (*Procyon lotor*) were trapped in residential areas by DeKalb County Animal Control personnel from March to June 2002, as part of the county's nuisance animal abatement program. After animals were trapped, they were returned to the animal control facility, euthanized, and necropsied. The small intestine was removed, split open, and visually examined for *B. procyonis* worms. A stool sample was collected, placed in 10% formalin, returned to the Centers for Disease Control and Prevention (CDC) laboratories (where it was processed by using a standard formalin ethyl-acetate concentration procedure), and examined microscopically for *B. procyonis* eggs.

Of 50 raccoons examined during the spring of 2002, a total of 24 were female and 26 were male; all were adults. Eleven (22%) animals (5 female, 6 male) were found to be infected with *B. procyonis*. All 11 animals had detectable eggs in the feces. Worms were recovered from the small intestine in 8 of these. The number of worms recovered from individual animals ranged from 1 to 24, with a mean of 6.25 worms per animal; all worms were preserved.

## Conclusions

Populations of raccoons harboring *B. procyonis* in and around major urban areas hold particular potential for zoonotic spread to humans. One reason is that raccoons adapt readily to human habitation and therefore tend to defecate in close proximity to homes (11), potentially putting large numbers of infective eggs in the immediate environment of children and others playing or working in yards, parks, playgrounds, and other similar environments. Heavily infected raccoons may shed millions of eggs daily, which is important because much human exposure to *Baylisascaris* is through the fecal-oral route and depends on the number of eggs in the environment (4,11,12).

To date, 12 human cases of infection with *B. procyonis* have been documented, and undoubtedly many more have been unrecognized. Four (30%) of these patients died, and the other patients were left with severe mental impairment. Most recognized cases, 10 of 12, have occurred in children 9 months to 6 years of age (13).

Until zoonotic diseases such as toxocariasis were actively sought with good serologic assays, their true occurrence was underestimated. The prevalence of *Baylisascaris* is undoubtedly greater than the number of reported cases would suggest, and the fact that the full clinical spectrum of illness for *Baylisascaris* infection has not been clearly elucidated, further lead to underrecognition of cases. Earlier case reports of diffuse unilateral subacute neuroretinitis or eosinophilic meningoencephalitis are compatible with *Baylisascaris* infection (4).

We cannot explain why *Baylisascaris* infection has turned up in the metropolitan Atlanta area at this time.

Historically, the infection has been absent from this region of the southeastern United States, and surveys, including a number of our own unpublished observations in northern and southern areas of Georgia over the past 10 years, have never encountered this infection (5). During the preparation of this report, however, we received a call from a licensed wildlife rehabilitator who had received several young raccoons from Athens (Clarke County), Georgia, one of which had passed *Baylisascaris* worms. This person was well trained and very cognizant of *Baylisascaris* but had never seen the infection in any animals until this animal was received in June 2002. Geographic movement of other infectious diseases has been well documented; it is often linked to legal or illegal movement of natural host animals for a variety of purposes. We have no evidence of recent, large-scale movement of raccoons from enzootic areas into the metropolitan Atlanta area, but other explanations seem implausible. This may represent a natural migration of the parasite into new areas, but, again, no explanation of why this would be happening at this time is obvious.

This report highlights for clinicians and other public health officials, especially in the southeastern United States, the potential occurrence of *Baylisascaris* in an area previously thought to not be at risk and the need to be alert to the possibility of *Baylisascaris*-induced encephalitis, especially in young children. The outcome of this infection in humans is often fatal, but if the infection is recognized and treatment initiated early, larvae may be killed before they enter the central nervous system, thus mitigating the clinical disease or preventing death (14).

### Acknowledgments

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Dr. Eberhard is director, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention. He has broad interests in the biology of parasitic infections, and his research interests include the epidemiology and diagnosis of zoonotic infections.

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Address for correspondence: Mark L. Eberhard, Division of Parasitic Diseases, Centers for Disease Control and Prevention, 4770 Buford Hwy, N.E., Atlanta, GA 30341-3724 USA; fax: 770-488-7794; email: mle1@cdc.gov

# Scrub Typhus Reemergence in the Maldives

**Michael D. Lewis,* Abdul Azeez Yousuf,† Kriangkrai Lerdthusnee,* Ahmed Razee,‡ Kirkvitch Chandranoi,* and James W. Jones***

In summer 2002, an outbreak of febrile illness began in the Maldives in the Indian Ocean. Through April 2003, officials recorded 168 cases with 10 deaths. The Armed Forces Research Institute of Medical Sciences in Bangkok confirmed *Orientia tsutsugamushi* and conducted a joint investigation with the Ministry of Health, Maldives. These cases of scrub typhus were the first in the Maldives since World War II.

In the summer of 2002, an outbreak of febrile illness began in the Republic of Maldives, a nation of 26 coral atolls straddling the equator in the Indian Ocean. The Ministry of Health, Republic of Maldives, intensified surveillance efforts, and scrub typhus was clinically suspected by the beginning of September. From May 28, 2002, through April 17, 2003, the Ministry of Health has recorded 168 suspected and confirmed cases with 10 deaths. The disease appears to have a focus (74 cases) in the Gaafu Dhaalu Atoll, just north of the equator, including 57 cases and three deaths on Gadhdhoo Island (year 2000 population = 1,719) (Table 1, Figure).

The last cases of scrub typhus in the Maldives were recorded by British troops during World War II (1). Following their arrival in October 1941 on Gan Island, Addu Atoll, the Royal Marines suffered an outbreak of 42 cases. In 1942, the British had another 582 cases, 382 in 1943, 92 in 1944, and none in 1945. J.R. Audy, a British Army physician during the war, reported that rats were numerous in the area and *Trombicula* (now *Leptotrombidium*) *deliense* were found in a variety of habitats (1). Scrub typhus was also documented by an Indian Army survey after the war (2). According to S.L. Kalra, General Headquarters–India raised a field typhus team that joined the Scrub Typhus Research Laboratory under the South East Asia Command in 1945. After the war, the team continued investigations of rickettsial diseases, surveying 23 locations from Addu Atoll, Maldives, to the Himalayas. A published report noted that beech rain forests in the Addu Atoll possessed both the host and vector of scrub

Table 1. Total cases, laboratory-confirmed cases, and deaths in the Maldives by atoll[a] and island (listed from north to south) from May 28, 2002, to April 27, 2003[b]

| Atoll | Island | Total cases | Laboratory-confirmed cases[b] | Deaths |
|---|---|---|---|---|
| Haa Alifu | Thakandhoo | 1 | 0 | 0 |
| | Filladhoo | 1 | 1 | 1 |
| | Naivaadhoo | 1 | 0 | 0 |
| | Baarah | 1 | 0 | 0 |
| | | **4** | **1** | **1** |
| Haa Dhaalu | Hanimaadhoo | 1 | 0 | 0 |
| | Nolhivaranfaru | 1 | 0 | 0 |
| | Kurinbi | 1 | 0 | 0 |
| | Vaikaradhoo | 2 | 0 | 1 |
| | | **5** | **0** | **1** |
| Shaviyani | Kanditheem | 2 | 1 | 1 |
| | Noomaraa | 1 | 0 | 0 |
| | Maroshi | 1 | 0 | 0 |
| | | **4** | **1** | **1** |
| Raa | Alifushi | 1 | 0 | 0 |
| | Rasgetheem | 2 | 0 | 1 |
| | Inguraidhoo | 10 | 0 | 1 |
| | Kinolhas | 1 | 0 | 0 |
| | | **14** | **0** | **2** |
| Baa | Thulhaadhoo | **2** | **0** | **0** |
| Kaafu | Malé | **4** | **0** | **0** |
| North Alifu | Rasdhoo | 3 | 0 | 0 |
| | Ukulhas | 2 | 2 | 0 |
| | Feridhoo | 4 | 1 | 1 |
| | | **9** | **3** | **1** |
| South Alifu | Himendhoo | **1** | **0** | **0** |
| Dhaalu | Rinbudhoo | 2 | 1 | 0 |
| | Gemendhoo | 1 | 1 | 0 |
| | | **3** | **2** | **0** |
| Thaa | Vilufushi | 1 | 0 | 0 |
| | Dhiyamigili | 1 | 0 | 0 |
| | Guraidhoo | 2 | 0 | 0 |
| | | **4** | **0** | **0** |
| Laamu | Isdhoo | 1 | 0 | 0 |
| | Gan | 1 | 0 | 0 |
| | Kadhdhoo | 2 | 0 | 0 |
| | | **4** | **0** | **0** |
| Gaafu Alifu | Viningili | 4 | 0 | 0 |
| | Koodhoo | 1 | 0 | 0 |
| | Dhaandhoo | 6 | 1 | 0 |
| | Maamendhoo | 1 | 0 | 0 |
| | Nilandhoo | 3 | 0 | 0 |
| | Gemanafushi | 4 | 1 | 1 |
| | Kanduhulhudhoo | 2 | 0 | 0 |
| | | **21** | **2** | **1** |
| Gaafu Dhaalu | Thinadhoo | 4 | 3 | 0 |
| | Maathadaa | 4 | 0 | 0 |
| | Gadhdhoo | 57 | 12 | 3 |
| | Vaadhoo | 2 | 1 | 0 |
| | Fiyoaree | 4 | 0 | 0 |
| | Fares | 5 | 0 | 0 |
| | | **76** | **16** | **3** |
| Gnaviyani | Fuvahmulah | **9** | **0** | **0** |
| Seenu (Addu) | Hithadhoo (Gan) | 5 | 0 | 0 |
| | Hulhudhoo | 1 | 0 | 0 |
| | Feydhoo | 2 | 0 | 0 |
| | | **8** | **0** | **0** |
| Total | | **168** | **38** | **10** |

[a]Numbers in bold indicate atoll totals.
[b]Confirmed either at the Armed Forces Research Institute of Medical Sciences (see Table 2) or by enzyme-linked immunosorbent assay at Indira Gandhi Memorial Hospital in Malé.

*Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand, †Ministry of Health, Malé, Republic of Maldives; and ‡Indira Gandhi Memorial Hospital, Malé, Republic of Maldives

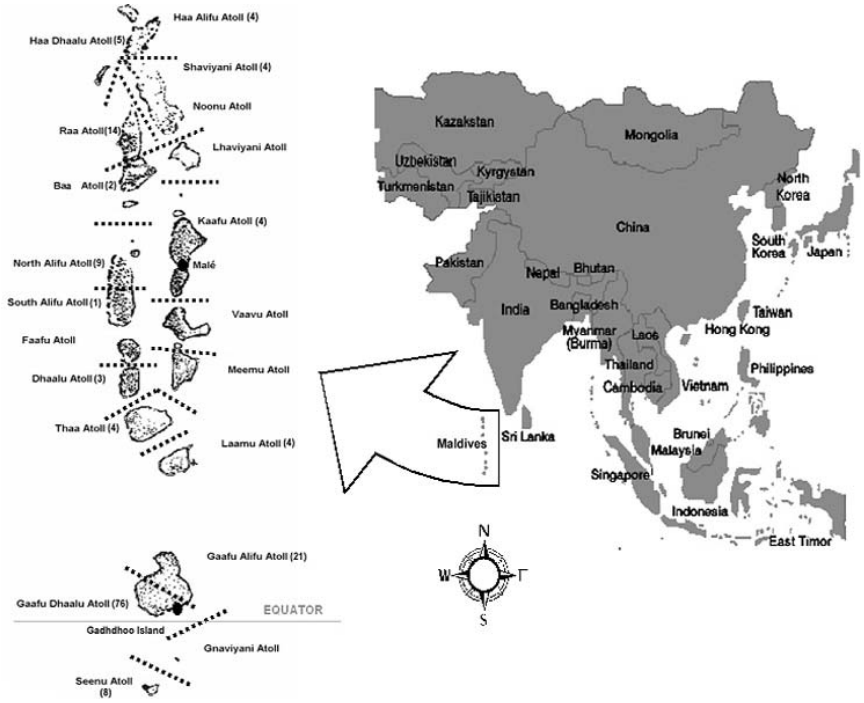


Figure. Republic of Maldives by atoll (total number of cases, May 28, 2002, to April 27, 2003, in parentheses next to atoll name).

typhus and the rickettsia, whereas the atoll was negative for Q fever and murine, epidemic, and tick typhus (2).

Scrub typhus is a chigger-borne disease caused by the rickettsia *Orientia tsutsugamushi* (3–6). The disease is endemic in Asia and remains an important public health problem (1,3–6). Scrub typhus was the most notable rickettsiosis affecting U.S. troops and had a higher mortality rate than any other infectious disease in WWII in the China-Burma-India theatre of operations (5). Larval trombiculid mites become infected with *O. tsutsugamushi* during feeds on their usual hosts, small rodents. Rodents influence mite population density and serve as a reservoir for the agent, although transovarial vertical transmission of the agent in mites is the dominant maintenance mechanism. Humans become infected when they accidentally encroach in an area where the chigger-rodent cycle is occurring, most often areas of low-lying scrub brush or transitional vegetation. *O. tsutsugamushi* is transmitted to humans when a chigger attaches itself to the skin in the search for food (3–7).

Scrub typhus often appears as a nonspecific febrile illness. Diagnosis and surveillance can be challenging, particularly in the absence of advanced laboratory diagnostic techniques (3–6). Typical symptoms include fever, headache, rash, and lymphadenopathy (3–7). The presence of an eschar is pathognomonic, but it is typically overlooked or misdiagnosed (3,4,6). Pulmonary involvement frequently occurs in mild cases and is the principal cause of death in severe disease (3,4). Before antimicrobial drugs, case-fatality rates ranged from 5% to 40% (3–7).

Since the departure of the British, no cases of scrub typhus have been recorded in the Maldives. Following the deaths of three adolescents on Gadhdhoo Island in July–August 2002, the Director General of Health Services, Ministry of Health, contacted researchers at the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, for assistance with diagnostics and disease control. In September 2002, AFRIMS received specimens from 31 patients: 28 serologic and 4 whole blood samples (both serum and whole blood received from one patient). Serologic testing was done by indirect fluorescent antibody (IFA), indirect immunoperoxidase (IIP), and enzyme-linked immunosorbent assay (ELISA) testing. The presence of the *O. tsutsugamushi* DNA was tested for by polymerase chain reaction (PCR) in the whole blood samples received.

Procedures for the IIP assay were those described by Suto (8) and Land et al. (9). The IFA was modified from the microimmunofluorescence methods described by Bozeman and Elisberg (10) and Robinson et al. (11). PanBio scrub typhus immunoglobulin (Ig) M and IgG rapid immunochromatographic assays were obtained (PanBio, Brisbane, Australia) and used according to the insert (Insert-2-SCT-25S, 13 July 2000). AFRIMS identified 14 of 28 serologic samples as positive for *O. tsustugamushi* infection (Table 2).

PCR is considered the most sensitive and specific method available for detecting the rickettsial DNA of *O. tsustugamushi* (13,15). Primary PCR and nested PCR were used for DNA amplification of *O. tsutsugamushi* (13).

AFRIMS identified *O. tsutsugamushi* DNA in two of the four whole blood samples provided.

At the invitation of the Minister of Health, Republic of Maldives, AFRIMS researchers conducted a joint consultation with health officials in the South Huvadhu Atoll and Malé, the capital. Assessments were conducted as well as meetings with the communities, community leaders, and healthcare workers involved in controlling the disease. Clinical, diagnostic, and entomologic advice was presented to healthcare providers at the Indira Gandhi Memorial Hospital in Malé in addition to providing the hospital laboratory with training and supplies to conduct ELISAs.

An onsite evaluation showed that while Gadhdhoo is mostly residential, rodent habitats exist. Wood for cooking fuel is commonly stored in backyards near kitchens, which are often outdoors. An island clean-up campaign to consolidate trash sites into two established locations appears to have been successfully implemented by the island leaders, but some yards still are prime rodent habitats, along with several common areas of low-lying transitional grassy areas. Many residents travel by boat several hundred meters to the uninhabited Gan Island to cultivate food such as yams. Gan Island is a textbook example of a chigger habitat. Trails leading to cultivated areas are bordered by tall grass and brushy habitat. Islanders reported that Gan Island was inhabited many years ago, but a mysterious fatal illness fell over the island and caused the residents to flee to Gadhdhoo decades ago.

Because health officials and the island leadership have been proactive in controlling the scrub typhus situation through rodent control and vegetation clean-up on Gadhdhoo Island, only two rodents were trapped (14) by local officials, both which were identified by AFRIMS as *Rattus remotus*. One rat was found to have 15 chiggers in the ear that were identified as *L. deliense* but were found not to be harboring *O. tsutsugamushi* when tested by PCR.

Three questions remain concerning the current outbreak of scrub typhus in the Maldives: 1) where did the scrub typhus come from; 2) why is scrub typhus now occurring; and 3) why is there a disproportionate focus on Gadhdhoo? Because of the transovarian and transtadial transmission of *O. tsutsugamushi* in mites (3–7), this pathogen has likely always been in the Maldives, but cases have been unrecognized since the British wartime occupation because of a lack of surveillance and diagnostics infrastructure.

The second and third questions are closely related. Scrub typhus, at least in Japan, can appear in cycles and completely disappear in between (6). Scrub typhus in the Maldives was known to occur in the 1940s, quite possibly due to the impact of introducing naïve British soldiers into a wartime occupational situation with a large intrusion on the natural habitat of rodents and chiggers (1). Not until the summer of 2002, when three adolescent deaths occurred on Gadhdhoo Island, was confirmatory laboratory diagnosis sought. The Ministry of Health then began educating healthcare providers and making the public aware of the situation.

What was different on Gadhdhoo Island that caused its population to suffer a disproportionate number of cases? In addition to the occurrence of a possible scrub typhus cycle, a large die-off of wild and domestic cats occurred in 2000. Cats are the small island's only natural predator of rodents. A subsequent large increase in the rodent population occurred over the next 2 years, leading to an increased interaction between the human and rodent populations. Additionally, in February 2002, the island leaders began an aggressive campaign to clean up trash sites and yards. Increased exposures to rodent populations as a result of disturbances of rodent habitats are well known to result in increases in the incidence of scrub typhus (1,3–6).

Through a combined effort between the Ministry of Health and AFRIMS, we have documented the reappearance of scrub typhus in the Maldives 58 years after the last reported cases. While the disease has possibly existed in the Maldives for many years, it is now being clinically recognized and diagnosed through the efforts of the Ministry of Health.

Table 2. Testing results performed at the Armed Forces Research Institute of Medical Sciences

| | IIP[a] | IFA[b] | ELISA[c] |
|---|---|---|---|
| IgM (+) | 14 | 15 | 14 |
| IgG (+) | 24 | 24 | 24 |
| IgM (+) + IgG (+)[d] | 14 | 15 | 14 |
| Total samples tested | 28 | 28 | 28 |
| PCR[e] and agarose gel electrophoresis positive results: | | | 2 of 4 |

[a]Indirect immunoperoxidase test (positive if titer was ≥400) (12).
[b] Indirect immunofluorescent antibody test (positive if titer was ≥50) (12).
[c]Enzyme-linked immunosorbent assay (positive if PanBio units >11) (12).
[d]Positive immunoglobulin (Ig) M plus IgG results indicate active *Orientia tsustugamushi* infection. IgG without IgM indicates the persistence of IgG after a primary infection but with no active infection. The absence of both IgG and IgM indicates no exposure or a very early stage of infection (12).
[e]Polymerase chain reaction for *O. tsustugamushi* DNA (13).

Dr. Lewis is a U.S. Army public health physician stationed in Bangkok, Thailand. He is responsible for the Department of Defense's Emerging Infections Program at the Armed Forces Research Institute of Medical Sciences and works with partners in Asia to develop disease surveillance and outbreak response systems.

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Address for correspondence: Michael Lewis, USPS Address—USAMC-AFRIMS, APO AP 96546 USA; fax: +662 644 4980; email: michael.lewis14@us.army.mil

# *Chlamydophila abortus* Pelvic Inflammatory Disease

**Gernot Walder,* Herwig Meusburger,† Helmut Hotzel,‡ Albrecht Oehme,§ Walter Neunteufel,† Manfred P. Dierich,* and Reinhard Würzner***

We report the first documented case of an extragestational infection with *Chlamydophila abortus* in humans. The pathogen was identified in a patient with severe pelvic inflammatory disease (PID) by sequence analysis of the *ompA* gene. Our findings raise the possibility that *Chlamydiaceae* other than *Chlamydia trachomatis* are involved in PID.

*Chlamydophila (Cp.) abortus*, whose strains are nearly 100% conserved in ribosomal and *ompA* genes, has recently been derived as new species from *Cp. psittaci* (1). It is the causative agent of enzoonotic abortion, which is frequently observed among sheep flocks in the eastern Alps and worldwide (2). By producing spontaneous abortion, stillbirth, or delivery of weak lambs, it is a major cause of reproductive failure in most sheep-rearing countries and, consequently, a serious economic problem (3).

*Cp. abortus* has also been characterized by serologic testing or sequence analysis from abortion in a horse, rabbit, pig, guinea pigs, and mice (1). It was first isolated from products of a septic human abortion in 1967 (4). Previously, human infections have been reported anecdotally (5), and *Cp. abortus* has been confirmed as the causative agent of septic abortion by ultrastructural and genetic analysis of isolates from women with previous contact with sheep (6–8). In humans, extragestational manifestations of infection with *Cp. abortus* have never been described. We therefore report the case of a 39-year-old woman with severe pelvic inflammatory disease (PID) caused by *Cp. abortus*.

## Case Report

In February 2001, a 39-year-old woman was admitted to the district hospital of Dornbirn, Vorarlberg, Austria, for chronic abdominal pain, increased vaginal discharge, and unusually heavy menses. Her medical record showed two uncomplicated pregnancies, followed by an aseptic abortion in the second trimester, a further uncomplicated pregnancy, and two first-trimester miscarriages. Since adolescence, the patient had experienced lower abdominal complaints, including menstrual irregularities, urinary symptoms, and unspecific vaginal discharge. Repeated treatment of mycosis and infections of the urinary tract did not lead to substantial clinical improvement nor did symptomatic treatment with corticosteroids or with *Lactobacillus acidophilus*. Her condition was exacerbated after a copper-containing intrauterine device was inserted in 1999. Episodes of lower abdominal pain became more frequent and more severe. They were accompanied by fatigue, general malaise, and sometimes by elevated temperature. Her menses became increasingly heavy, finally resulting in 10 days of heavy bleeding. Clinical investigation on admission showed lower abdominal tenderness, cervical motion tenderness, and bilateral adnexal tenderness, more prominent on the right. The patient had signs of compensatory hypochromic anemia, which was attributable to menorrhagia. Erythrocyte sedimentation rate (22–44 mm/h) and C-reactive protein (0.6 mg/dL) were moderately elevated. Leukocyte count was normal as was the patient's oral temperature. Results of tests for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* by ligase chain reaction (LCx; Abbot Laboratories, Vienna, Austria) were negative.

Because chronic PID was suspected, the intrauterine device was removed. Diagnostic pelviscopy indicated diffuse coalescence of both adnexes and edematous swelling of the fallopian tubes with severe postinflammatory changes (Figure). Amber liquid was extracted from the pouch of Douglas for further microbiologic investigation. Aerobic and anaerobic cultures remained sterile as did cultures for *Ureaplasma urealyticum* and *Mycoplasma hominis* on special media (Biomerieux, Nürtingen, Germany). Results of LCx tests for *C. trachomatis* and *N. gonorrhoeae* were negative, and notable levels of antibodies against a constant region of the major outer membrane protein of *C. trachomatis* were not found in the patient's serum by enzyme-linked immunosorbent assay (ELISA) (Medac, Hamburg, Germany). However, the patient showed high levels of antibodies against genus-specific lipopolysaccharide of *Chlamydiacaea* (LPS-ELISA, Medac). Thus, microimmunofluorescence assays (MIF) for *C. trachomatis*, *Cp. pneumoniae* (both in house MIF Jena) and *Cp. psittaci* (Biomerieux, Nürtingen, Germany) showed high antibody titers against *C. psittaci* (1:512), titers against *Cp. pneumoniae* were within the normal range (1:16), and the result of the *C. trachomatis* reaction was unspecific and interpreted as negative. A retrospective analysis found that notable levels of antibodies against the

*Institute of Hygiene and Social Medicine, University of Innsbruck, Innsbruck, Austria; †District Hospital of Dornbirn, Dornbirn, Austria; ‡Federal Institute for Consumer Protection and Veterinary Medicine, Jena, Germany; and §Friedrich-Schiller-Universität Jena, Jena, Germany

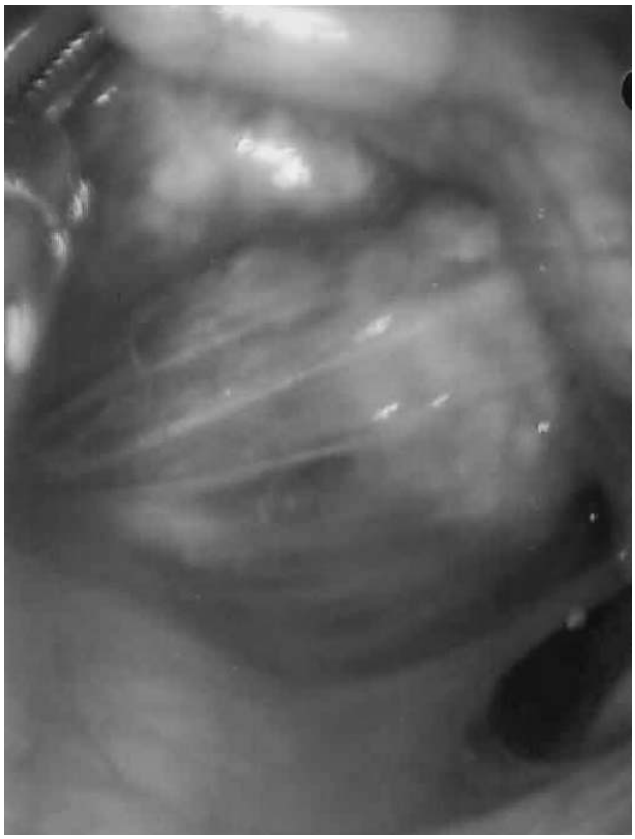

Figure. Diffuse coalescences between uterus and fallopian tubes (the ovary is hidden behind the coalscences).

heat-shock protein 60 (hsp60) were demonstrated in the patient's serum by ELISA. Nested polymerase chain reaction (PCR) for *Chlamydiaceae* spp.–specific *ompA* (9) was done from the pouch of Douglas liquid and yielded a positive result.

Sequence analysis of the resulting PCR product showed that it had the highest homology to *Cp. abortus*. The isolate's *ompA* gene region was >99% homologous with *Cp. abortus* strains, whereas *Cp. psittaci* was <90% homologous.

The patient was treated with doxycycline (Vibravenoes, 200 mg–100 mg for 5 days). Six weeks later, all laboratory parameters were within normal ranges, the patient's fatigue had subsided, and she had not experienced further episodes of elevated temperature. Tenderness of the lower abdomen had subsided, except for a slight monolateral adnexal tenderness on the right side, consistent with a decreased swelling of the right fallopian tube shown by sonogram. Eighteen weeks later, both adnexes and the lower abdomen were indolent upon palpation, the patient felt well, and a sonogram showed a further regression of the swelling of the right fallopian tube. When questioned 9 months after treatment, the patient did not report any lower abdominal symptoms, and her menstrual irregularities had subsided.

## Discussion

The patient had likely been infected with *Cp. abortus*. This conclusion is supported by the positive result of the PCR from Douglas liquid and the sequence analysis of the *ompA* gene. Because *Cp. abortus* is highly infectious, it requires C3 equipment for culturing; thus, no attempt was made to confirm this result by culture. The serologic investigation provided further support for the involvement of *Cp. abortus* in this case: High antibody titers to LPS indicate chronic infection or, less likely, multiple expositions to a member of the genus *Chlamydiaceae* (10). The high immunofluorescence assay titer to *Cp. psittaci* is consistent with an infection with *Cp. abortus* because both species are closely related and share most surface proteins (11), making a serologic distinction between both pathogens virtually impossible. The absence of antibodies specific to *C. trachomatis* and the repeatedly negative results of the ligase chain reaction for detection of *C. trachomatis* exclude a concomitant infection with this pathogen. A careful search for other microorganisms in the patient's Douglas extract did not yield a pathologic result.

Infection with *Cp. abortus* has hitherto exclusively been reported in pregnant women, beginning as an influenzalike illness with consecutive development of thrombocytopenia and coagulopathy, usually resulting in fetal death (4,5). Symptomatic carriers have been described in sheep, with the pathogen being shed in periovulatory estrus (12), but the possibility of chronic infection or the possibility of extragestational illness has never been evaluated in humans.

In light of previous investigations of chronic infection with *C. trachomatis, Cp. abortus* appears to be a probable PID. Salpingitis and postinflammatory adhesions as observed in our patient are known sequelae of genital chlamydial infection in animals and humans (13). Some evidence shows that inflammation and subsequent tissue damage in chronic PID are due to an immunopathologic reaction against a chlamydial heat-shock protein (hsp60) (14). Heat-shock proteins are highly conservative. A high amino acid identity exists between the hsp60 of *C. trachomatis* and the hsp60 of other *Chlamydiaceae* (*Cp. caviae,* 93%; *Cp. pneumoniae*, 80%) as well as stress response proteins found in other microorganisms (15). Identity to the htpB protein of *Coxiella burnetii* is 61%, to the GroEL protein of *Escherichia coli* it is 60%, and to human HuCha 60, 48% (15). That the hsp60-specific antibodies in our patient's serum were induced by *Cp. abortus* is highly likely, and this genus can cause PID in a way similar to that proposed for *C. trachomatis*. Thus, we conclude that *Cp. abortus* has to be considered in patients with PID disease and should be ruled out with suitable diagnostic methods. When PCR is applied, the preferred method

should amplify sequences shared by all members of *Chlamydiaceae*.

The exacerbation of our patient's symptoms after the insertion of the intrauterine device was striking, and the possible underlying mechanism needed to be considered. A coincidental infection with the pathogen at the same time was unlikely because no evidence for that was found in the patient's medical history, and the serologic results pointed towards chronic infection. However, the patient reported extensive contact with sheep and other ruminants in her youth, which she terminated due to bronchopulmonary complaints. Whether they were due to an allergic reaction or an infection of the lower airways remained unclear. Her unspecific genecologic symptoms started at approxymately the same time, about 2 years after her menarche and about 5 years before her first pregnancy. Exact data on how the expression of hsp60 in *Chlamydia* is influenced by copper are not available. Limited evidence has indicated that copper induces the expression of hsp60 in rotifers (16) and that copper deficiency reduces the expression of hsp60-like proteins in rats (17). Because the amino acid sequence of the heat-shock protein and the amino acid sequence of the promotor region are highly conservative (15), we propose that the intrauterine device might have induced the expression of hsp60, which became the source of antigenic stimulation for an autopathologic immune response. Consistent with this theory is the finding that our patient's symptoms subsided after the intrauterine device was removed and the chlamydial infection had been treated with antimicrobial drugs. Some evidence indicated that the chlamydial organisms are required in order for chronic PID to develop (18), and although information about the use of antimicrobial drugs in chronic stages of PID is limited, they have proven effective in other *Chlamydia*-triggered autoimmune diseases (19). Further studies are under way to investigate the clinical importance of extragestational infection with *C. abortus* and the influence of copper on the expression of stress response proteins in *Chlamydiaceae*.

Dr. Walder is responsible for the laboratory of serologic investigations at the Institute of Hygiene and Social Medicine at the University of Innsbruck, Austria. His research interests include zoonotic diseases and immunopathologic reactions.

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Address for corresondence: Gernot Walder, Unterwalden 30, A-9931 –Ausservillgraten, Austria; fax: (0043)-(0)512-578745; email: gernot.walder@uibk.ac.at

# Influenza Pandemic Preparedness

**Kathleen F. Gensheimer,* Martin I. Meltzer,† Alicia S. Postema,† and Raymond A. Strikas†**

In the list of potential bioterrorist agents, influenza would be classified as a category C agent (1). While previous influenza pandemics were naturally occurring events, an influenza pandemic could be started with an intentional release of a deliberately altered influenza strain. Even if a deliberately altered strain is not released, an influenza pandemic originating from natural origins will inevitably occur (2) and will likely cause substantial illness, death, social disruption, and widespread panic. Globally, the 1918 pandemic killed at least 20 million people (3). This figure is approximately double the number killed on the battlefields of Europe during World War I (4). In the United States alone, the next pandemic could cause an estimated 89,000–207,000 deaths, 314,000–734,000 hospitalizations, 18–42 million outpatient visits, and 20–47 million additional illnesses (5). These predictions equal or surpass many published casualty estimates for a bioterrorism event (6–8). In addition to the potential for a large number of casualties, a bioterrorism incident and an influenza pandemic have similarities that allow public health planners to simultaneously plan and prepare for both types of emergencies (Table).

Preparing for both the next influenza pandemic and the next bioterrorist attack requires support and collaboration from multiple partners at the state, local, and federal level. Potential partners include the medical community, law enforcement, emergency management, and public health agencies. To help foster these crucial cross-discipline relationships, the Centers for Disease Control and Prevention (CDC) and the Council of State and Territorial Epidemiologists (CSTE), in collaboration with the National Emergency Management Association, the Association of State and Territorial Health Officials, the Federal Emergency Management Agency, and the Association of Public Health Laboratories, hosted a 2-day meeting on state and local pandemic influenza planning in May 2002. Over 125 officials representing epidemiology, communicable disease, laboratory, immunization, and emergency management programs from 46 states registered for this meeting. The objectives of the meeting were to enhance collaboration between state and local public health and emergency management agencies, establish mechanisms for integrating bioterrorism and pandemic influenza preparedness and response planning, and develop policy and strategy options for influenza pandemic preparedness and response at the state and local level. We report the results of a questionnaire distributed to the attendees; it was designed to elicit their views on the most important issues that must be addressed by a plan to respond to a catastrophic disease event.

## Priorities for Pandemic Influenza Planning

All plans for any catastrophic infectious disease event such as pandemic influenza or a bioterrorist attack must address five topics: surveillance and laboratory issues; communications; maintenance of community services; medical care; and supply and delivery of vaccines and drugs. After presentations providing background information, conference attendees were divided into breakout groups to discuss these topics. The groups did not discuss particular scenarios, but the presentations given before the breakout groups did include details of estimates of the potential impact of the next influenza pandemic (5). Attendees completed short (≤5 questions), anonymous questionnaires at both the beginning and end of the breakout session. Each breakout group had a different set of questions relevant to the topic of that group.[1] However, all groups addressed a common question, which asked persons to pick their top priority for a pandemic influenza response from one of the following options: reduce mortality, reduce morbidity, ensure continuation of essential services, reduce economic impact, and ensure equitable distribution of resources. As explained to the attendees before the breakout session, differences by age and risk group in rates of mortality and morbidity could mean that public health officials with limited resources might not be able to simultaneously maximize reductions in mortality and morbidity (5). The first three options were chosen most frequently (Figure). Even after discussion, no option was cho-

*Department of Human Services, Augusta, Maine, USA; and †Centers for Disease Control and Prevention, Atlanta, Georgia, USA

[1]A complete copy of each questionnaire and a complete set of the results are available from the corresponding author.

Table. Planning for pandemic influenza and bioterrorism: similarities and differences

| Issue | Bioterrorist event | Pandemic influenza |
|---|---|---|
| Likelihood | High | High |
| Warning | None to days | Days to months |
| Occurrence | Focal or multifocal | Nationwide |
| Transmission/duration of exposure | Point source; limited; person-to-person | Person-to-person, 6–8 wks |
| Casualties | Hundreds to thousands | Hundreds of thousands to millions |
| First responders susceptible? | Yes | Yes |
| Disaster medical team support/response | Yes | No (too widespread) |
| Main site for preparedness, response, recovery, and mitigation | State and local areas | State and local areas |
| Essential preparedness components | | |
| Surveillance | Yes | Yes |
| Law enforcement intelligence | Yes | No |
| Investigation | Yes | Yes |
| Research | Yes | Yes |
| Liability programs | Yes | Yes |
| Communication systems | Yes | Yes |
| Medical triage and treatment plans | Yes | Yes |
| Vaccine supply issues | Yes (for most likely threats) | Yes |
| Drug supply issues | Yes | Yes |
| Training/tabletop exercises | Yes | Yes |
| Maintenance of essential community services | Yes | Yes |
| Essential response components | | |
| Rapid deployment teams | Yes | No |
| Effective communications/media relations strategy | Yes | Yes |
| Vaccine delivery | Yes (for some) | Yes |
| Drug delivery | Yes (for most) | Yes |
| Hospital/public health coordination | Yes | Yes |
| Global assistance | Possibly | Yes |
| Medical care | Yes | Yes |
| Mental health support | Yes | Yes |
| Mortuary services | Yes | Yes |
| Supplies and equipment | Yes | Yes |
| Essential mitigation components | | |
| Enhanced surveillance | Yes | Yes |
| Enhanced law enforcement intelligence | Yes | No |
| Vaccine stockpile | Yes (selected agents) | Prototype vaccines only |
| Drug stockpile | Yes | Yes |
| Pre-event vaccination | Vaccination of selected groups[c] | Vaccination of groups at medical high risk with pneumococcal vaccine[d] |

[a]During a catastrophic infectious disease event, such as an influenza pandemic, there may be critical shortages of vaccines and drugs. Thus, clinics set up to administer vaccines and distribute antimicrobial drugs may require the services of a range of personnel whose fields of expertise are nonclinical. Examples of additional personnel that may be needed include law enforcement, translators, social workers, psychologists, and legal experts.
[b]Source: Adapted from: National Vaccine Program Office. Pandemic influenza: a planning guide for state and local officials (Draft 2.1). Atlanta: Centers for Disease Control and Prevention; 2000.
[c]At the time of writing, the smallpox vaccination program was just beginning. For other bioterrorist agents for which vaccines are available (e.g., anthrax), limited supplies and concerns about safety profiles have, up to this point, effectively prevented the widespread use of these vaccines.
[d]It may eventually be possible to vaccinate high-priority groups and the general population with a yet-to-be-developed "common epitope" vaccine, which might provide for a broader spectrum of protection against a variety of influenza A subtypes.

sen by >50% of attendees, indicating that this group of professionals did not have a unified opinion regarding what the top priority should be to guide planning and response measures.

Conference attendees did, however, agree that global and domestic laboratory and disease surveillance must be strengthened to increase the likelihood of early detection and tracking of either pandemic influenza or a bioterrorist event. A rise beyond the baseline number of influenza-like illnesses (ILIs) could indicate a severe influenza season, arrival of pandemic influenza, or early warning of a bioterrorist attack with a pathogen that causes ILIs (e.g., anthrax). Thus, the number and accuracy of reports of ILI, ILI outbreaks, and laboratory-confirmed reports of influenza need to be increased. In addition, ensuring that adequate laboratory and disease surveillance systems are in place will benefit the public health response during yearly influenza epidemics. Conference attendees identified two critical gaps in infectious disease surveillance systems: 1) less than ideal or nonexistent systems to monitor outpatient and hospital-based ILI cases and 2) insufficient numbers of laboratory personnel and epidemiologists to monitor, provide diagnostic support, and respond to events.

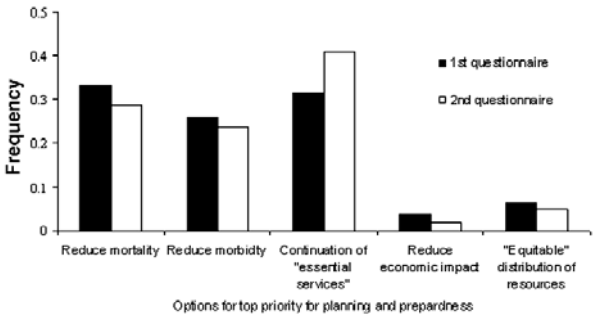


Figure. Distribution of responses identifying which goal should be the top priority for pandemic influenza planning and response (n = 107). During the conference, attendees were split into five groups for a breakout session. At the beginning and end of each such session, each attendee was given anonymous questionnaires. Each group had the same first question, in which attendees were asked to choose one of five options for top priority for influenza pandemic planning. This figure shows the frequency distribution of the attendees' choices.

Another critical component of any catastrophic infectious disease plan is communications. The anthrax attacks in 2001 demonstrated that the public, media, and healthcare professionals will demand accurate information, with frequent updates throughout the emergency. To minimize the potential for confusion, states and localities need to identify a recognized and trusted leader who will be the primary spokesperson to disseminate accurate information. Among attendees in the communications breakout group, 40% felt that the state governor would be the best spokesperson, 40% chose the state health officer, and 20% chose the state epidemiologist.

In the initial stages of, and potentially throughout, an influenza pandemic or a bioterrorist attack, there will be a shortage of many essential resources, including medical equipment and supplies, personnel, vaccines, and drugs. Prioritizing medical resources will therefore be necessary. The medical care breakout group unanimously chose state and local government as the authority that should prioritize and distribute healthcare resources. In the breakout group that discussed vaccine and antimicrobial agent issues, 73% chose essential workers and physicians as those who should be the first to receive vaccine and antiviral drugs. Only 27% chose those at high risk for adverse influenza-related health outcomes to be early recipients of vaccine.

## Conclusions: Maximizing Resources and Planning Efforts

Conference attendees were well aware of the need to simultaneously plan and prepare for the next influenza pandemic and the next bioterrorist event. However, much work remains to be done. Without agreement regarding the top priority for allocating scarce resources, planning and implementing an optimal response to either pandemic influenza or a bioterrorist event will be difficult, if not impossible. Illustrating potential planning problems was the incongruity between the inability of most attendees to agree on the goal of planning and response measures (Figure) while 75% of a subgroup stated that essential workers and physicians should be the first to receive vaccines and antiviral drugs. In a situation with limited resources, usually only one goal can be optimized (either maximized or minimized) (9). Therefore, before accepting any of the initially limited supplies of vaccine and antiviral drugs, physicians and first responders will have to explain how such an allocation will help achieve the chosen top priority.

Unprecedented resources for enhancing the public health preparedness and response infrastructure have been recently provided to all states by congressional appropriations in the form of bioterrorism cooperative agreements. The request for proposals stated that planning moneys may be used "…to upgrade state and local public health jurisdictions' preparedness for and response to bioterrorism, other outbreaks of infectious disease, and other public health threats and emergencies…" (10). Using such resources and reflecting upon the lessons learned from previous influenza pandemics and the 2001 terrorist events, public health, medical, and emergency management communities must work together to develop an effective plan to strengthen our national readiness to respond to any catastrophic infectious disease situation.

If our public health planning efforts are too narrowly focused on preparing responses to a few select bioterrorism-related scenarios, a new opportunity for planning responses to a broad spectrum of infectious disease-related catastrophes will be lost. Any plans made for responding to either pandemic influenza or bioterrorism events must include an explicit mechanism for making difficult decisions regarding the prioritization of scarce resources. The conference highlighted the need for all states to continue their discussions and public debates regarding the setting of priorities and methods for allocating scarce resources. Obviously, each state or local government will chose its own specific method for drawing up a plan to deal with catastrophic infectious disease events such as an influenza pandemic. To help aid the planning process, materials such as a planning guide are available from agencies such as CDC and CSTE. Ideally, such planning and prioritization activities should take place well in advance of any catastrophic infectious disease event.

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Dr. Gensheimer is the state epidemiologist and the director of the Medical Epidemiology Section, Maine Bureau of Health. She helps coordinate responses to outbreaks of disease as well as working on existing programs to promote the welfare and safety of the citizens of Maine. She has taken a leading role in influenza pandemic planning.

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Address for correspondence: Martin I. Meltzer, Mailstop D59, 1600 Clifton Rd., Atlanta, GA 30333, USA; fax: 404-371-5445; email: qzm4@cdc.gov

# Generalized Vaccinia 2 Days after Smallpox Revaccination

**To the Editor:** Hospital and public health personnel are currently receiving smallpox vaccination in a national effort to increase preparedness for a possible deliberate release of smallpox (1). Generalized vaccinia (GV) is a typically self-limited adverse event following vaccination (incidence 23.4–238.2 cases per million primary vaccinees and 1.2–10.8 cases per million revaccinees) (2,3).

We report the clinical course and laboratory diagnosis of GV in a 37-year-old woman with a history of at least one uncomplicated childhood inoculation that left a vaccination scar. She was revaccinated on March 12, 2003. Before revaccination, the patient reported no contraindications to vaccination and denied any conditions that typically weaken the immune system (including HIV/AIDS, leukemia, lymphoma, other cancers, radiation, chemotherapy, organ transplant, post-transplant therapy, immunosuppressive medications, severe autoimmune disease, and primary immune deficiency). The patient also confirmed that she did not have a skin disease or a history of eczema or atopic dermatitis, nor was she pregnant or allergic to a vaccine component.

On March 14, some 44 hours after vaccination, the patient reported headache, chills, pruritus, chest pain (described as chest "heaviness"), recurrent vomiting, and maculopapular lesions. The lesions, characterized by the patient as "mosquito bites," first appeared on the face, then the legs, and then the trunk and upper extremities. Maximum oral temperature was 37.7°C. Over the next 4 days, approximately 30 pustules developed, several of which began to drain. Nausea persisted, and the patient had a stiff neck and recurring chest tightness, but physical examination, echocardiography, electrocardiography, and chest radiography results were within normal limits. By March 25, the patient's lesions had all scabbed, the scabs had fallen off, and she felt well enough to return to work. Pustular material obtained on March 18 from two unroofed lesions on the shoulder (Figure) and back tested positive at the Wadsworth Center-Axelrod Institute, New York State Department of Health, for vaccinia virus DNA by a TaqMan (Applied Biosystems, Foster City, CA) real-time polymerase chain reaction assay provided by the Laboratory Response Network, Centers for Disease Control and Prevention. The presence of ortho-poxvirus was confirmed by electron microscopy of lesion fluid.

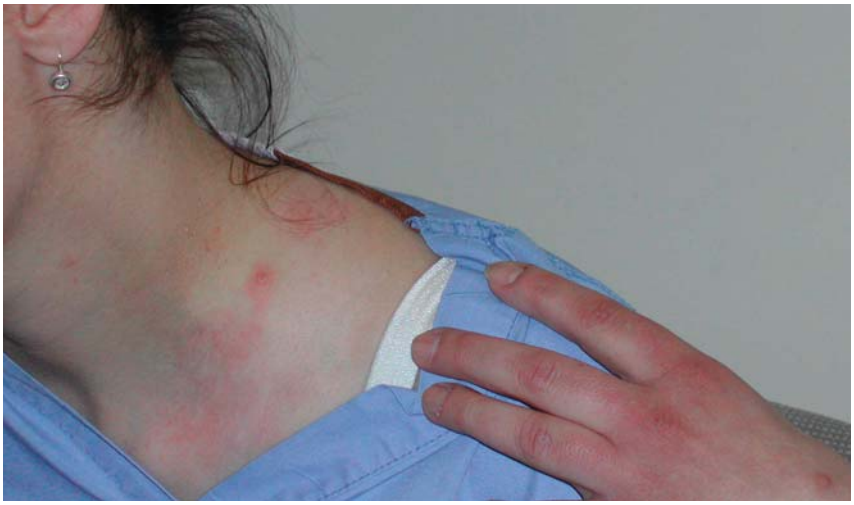

Figure. Pustular lesion on patient's shoulder, 6 days after revaccination.

This case is the first report of a laboratory-confirmed case of GV among recent civilian vaccinees and is notable for the GV occurrence in a revaccinee. GV was not reported among 132,656 military personnel recently revaccinated (4). A single case of GV in a revaccinee among 38,514 recent civilian vaccinations (5) yields a ratio that exceeds the rate in revaccinees observed in earlier reports and the difference would be even greater if civilians who received primary vaccinations were excluded.

This laboratory confirmation of GV demonstrates the potential of laboratory testing to determine the cause of a post-vaccination rash. Possible cases of GV in earlier surveillance efforts represented a mixed group of rashes, some of uncertain etiology (6). This patient's clinical course is notable for the onset of GV 2 days after vaccination, as compared to a mean of 9 days (range 1–20+) after (generally primary) vaccination (2) and suggests that viremia can occur quickly after vaccination.

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**James R. Miller,* Nick M. Cirino,* and Edward F. Philbin†**

*New York State Department of Health, Albany, New York, USA; and †Albany Medical College, Albany, New York, USA

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Address for correspondence: James R. Miller, New York State Department of Health, Bureau of Communicable Disease Control, Corning Tower Rm 678, Albany, NY 12237, USA; fax: 518-486-2249; email: jrm17@health.state.ny.us

## *Salmonella enterica* Serovar Enteritidis, Japan

**To the Editor**: Nontyphoidal salmonellae are the important causative agents of foodborne diseases in Japan and other industrialized countries. *Salmonella enterica* serovar Enteritidis has risen to the leading cause of infection among *Salmonella* spp. since 1989 (1). Emergence of drug-resistant *S*. Enteritidis has been rarely reported while *S*. Typhimurium, another serovar of major public health concerns, has been reported to acquire multidrug resistance such as DT104 resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline (R-ACSSuT) (2).

We previously reported outbreaks caused by strains resistant to ampicillin and streptomycin (resistance type R-AS, herein); the strains' reactions against the phages used in bacteriophage typing did not conform to any known reaction patterns (phage type [PT] RDNC-a, herein, with the following reactions: (-) for #3, 5–7, 11–13, 15, and 16 phages; (+++) for #2 phage; opaque lysis [OL] for #4 and 9 phages; <OL for #10 phage; and ambiguous reactions (-/+++) were observed for #1, 8, and 14 phages) (3). To investigate the characters of the R-AS strains more extensively, we surveyed isolates from outbreaks that occurred from 1997 to 2002 for antimicrobial drug susceptibility and bacteriophage typing.

*S*. Enteritidis strains from 899 outbreaks that occurred from 1997 to 2002 were tested. Bacteriophage typing was done according to the Public Health Laboratory Service (PHLS), London, United Kingdom guidelines (4). Antimicrobial drug susceptibility testing was done with a disc diffusion method on Mueller-Hinton II agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) as previously described (5). Antimicrobial drugs used in this study were ampicillin, streptomycin, tetracycline, kanamycin, nalidixic acid, gentamycin, sulfamethoxazole-trimethoprim, trimethoprim, chloramphenicol, cefotaxim, and ciprofloxacin.

Dominant phage types were PT4 (36.9%) and PT1 (26.9%). They have been dominant among outbreak-related strains since 1992 (1). Other types were also identified at certain frequencies. For example, RDNC-a, PT47, PT6, PT6a, and PT21 accounted for 4.4%, 5.3%, 4.0%, 3.2%, and 2.0% of the phage types, respectively.

Strains sensitive to all the antimicrobial drugs tested were the most predominant (55.1%), followed by those resistant to only streptomycin (34.8%). R-AS was the third most predominant, accounting for 4.1%. A correlation existed between drug resistance and phage types in that all the R-AS strains (n = 37) showed RDNC-a in bacteriophage typing, and all the RDNC-a strains (n = 40) were resistant to at least ampicillin including two R-A and one R-AST strains.

Since previous studies described the correlation between drug resistance and phage types as a result of acquisition of an R-plasmid (6), we focused on the relationship between RDNC-a and ampicillin resistance. Plasmid profiles analysis of the RDNC-a strains showed that all but one (R-AST) had at least two kinds of plasmids, and all but one were approximately 50 kb and 60 kb in size. The last could be the so-called serovar-specific plasmid (7). Southern blot analysis by using the ampicillin resistance gene of pBluescript KS (+) (Stratagene, La Jolla, CA) as a probe indicated that a resistance gene was carried on the 50-kb plasmid. Furthermore, when *Escherichia coli* DH10B cells (Invitrogen Corporation, Carlsbad, CA) were transformed with plasmids isolated from an RDNC-a R-AS strain and plated onto Luria broth plates containing 100 mg/L of ampicillin, the 50-kb, but not 60-kb, plasmid could be isolated from the ampicillin-resistant transformants. And the 50-kb plasmid from the transformants was hybridized to the probe for ampicillin resistance described above. Thus, the 50-kb plasmid of RDNC-a R-A or -AS strains was suggested to be an R-plasmid responsible for ampicillin resistance.

A representative 50-kb plasmid (p981123) was prepared from the DH10B transformant cells described earlier for further characterization. Southern blot analysis suggested that a 6-kb *Eco*RI fragment contained the resistance determinant. Sequences for the fragment were analyzed done by using ABI PRISM 310 sequencer and BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The resulting sequence showed high similarities to *Pseudomonas aeruginosa* Tn801 (accession no. AF080442; 98% identical) and *E. coli* Tn3 (accession

no. ISTN3X; 96% identical), comprising one of Tn3-like inverted repeats and putative coding regions for transposase, resolvase (also called repressor), and ampicillin resistance. The resistance gene encodes a TEM-1 type β-lactamase. (The sequence has been registered to DDBJ/GenBank/EMBL with accession no. AB103092.)

Conjugative transferability of p981123 between *S*. Enteritidis strains was examined by using the parental *S*. Enteritidis RDNC-a R-AS strain as a donor, and three independent *S*. Enteritidis strains (PT1; PT4; and PT21) resistant to nalidixic acid (R-N) as recipients. p981123 was transferable between *S*. Enteritidis strains at frequencies of $10^{-5}$ to $10^{-4}$, and the resulting R-AN transconjugant showed the same lytic pattern of the typing phages as RDNC-a. Thus, transfer of p981123 could convert the phage types at least from PT1, PT4, and PT21 to RDNC-a. Pulsed-field gel electrophoresis (PFGE) was done by using *Xba*I or *Bln*I as well, and RDNC-a strains showed a variety of PFGE profiles. These results suggest emergence and prevalence of the 50-kb R-plasmid converting phage types to RDNC-a in *S*. Enteritidis in Japan.

Previous studies reported correlation between R-plasmids and phage types of *S*. Enteritidis, where, for example, a 34-MDa R-plasmid of incompatibility group N (IncN) (8) and a 36-MDa R-plasmid of IncX (pDEP57) (6) were described. Both kinds of plasmids encoded ampicillin resistance as well as that in this study, but both were identified in PT6a isolates. Preliminary sequence data of the region of p981123 essential for replication indicated a gene coding for a protein similar to protein p1 of R6K (IncX) plasmid (9), which suggests that p981123 may be related to pDEP57. However, the reactions to the typing phages in RDNC-a strains were different from those in PT6a. Therefore, the R-plasmid in this study seems to have different features from previous ones. In addition, *S*. Enteritidis PT6d resistant to ampicillin was recently reported (10). Relationship between RDNC-a in this study and PT6d is unknown, and further investigations will be needed.

Transfer of an R-plasmid is a common way for bacteria to acquire drug resistance, and it often affects other aspects such as sensitivity of bacteriophages, as described in this study. Molecular based surveillance for drug resistance in *S*. Enteritidis needs to continue.

**Hidemasa Izumiya,* Naomi Nojiri,* Yoshiko Hashiwata,† Kazumichi Tamura,* Jun Terajima,* and Haruo Watanabe***

*National Institute of Infectious Diseases, Tokyo; and †Hiroshima City Institute of Public Health, Hiroshima, Japan

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Address for correspondence: Haruo Watanabe, Department of Bacteriology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan; fax: +81-3-5285-1171; email: haruwata@nih.go.jp

## Factors Influencing Fluoroquinolone Resistance

**To the Editor:** Recently, Scheld summarized factors that he considered to have an influence on the efficacy of fluoroquinolones (1). In the review, ciprofloxacin was presented as the most active fluoroquinolone against *Pseudomonas aeruginosa*

with MICs typically two- to eightfold lower than those for levofloxacin, moxifloxacin, or gatifloxacin. However, because the National Committee for Clinical Laboratory Standards (NCCLS) MIC interpretative breakpoints are fluoroquinolone-specific, percent susceptibility is considered to be a better measure by which to compare fluoroquinolone activities. Our company has conducted annual investigations called TRUST (Tracking Resistance in the United States Today) since 1996. These surveillance studies have consistently shown similar susceptibility rates for levofloxacin (67.7% in 2002) and ciprofloxacin (67.4% in 2002) against *P. aeruginosa* (2,3). Both agents show higher in vitro activity against *P. aeruginosa* than gatifloxacin and moxifloxacin (2–4). A critique of antipseudomonal fluoroquinolone activity should also consider peak achievable fluoroquinolone levels at a site of infection, the area under the serum concentration curve in 24 hours ($AUC_{24h}$), and the $AUC_{24h}$/MIC ratio (5). At equivalent dosages for nosocomial pneumonia, levofloxacin (750 mg intravenously, once daily) has a threefold higher peak serum level ($C_{max}$) and threefold higher $AUC_{24h}$ than ciprofloxacin (400 mg intravenously, every 8 hours) (package inserts for Levaquin and Cipro). While certain *P. aeruginosa* isolates have lower ciprofloxacin than levofloxacin MICs, the two fluoroquinolones have equivalent activity against *P. aeruginosa* because of their equivalent $AUC_{24h}$ /MIC ratios (6). We agree strongly with Scheld's suggestion that the fluoroquinolone used clinically should be the fluoroquinolone tested by the laboratory and reported; surrogate testing of fluoroquinolones may lead to major errors in reporting, particularly for *Enterobacteriaceae* (2,3,7).

The review also stated that levofloxacin-resistant strains of *P. aeruginosa* emerge at a significantly higher rate than with ciprofloxacin. However, a recent study of *P. aeruginosa* isolated from cystic fibrosis patients reported that fewer resistant mutants were isolated after exposure to levofloxacin (11 mutants) than to ciprofloxacin (28 mutants) (8).

With regards to *S. pneumoniae*, the review stated that in vitro studies have demonstrated that ciprofloxacin (1–4 mg/L) and levofloxacin (1–2 mg/L) are not as active as moxifloxacin (0.06–0.25 mg/L) and gatifloxacin (0.5–1 mg/L) against pneumococci. As with *P. aeruginosa*, fluoroquinolone comparisons against *S. pneumoniae* should not be limited to MICs alone because pharmacokinetic and pharmacodynamic characteristics differ for each fluoroquinolone. Pneumococcal time-kill studies with levofloxacin, gatifloxacin, and moxifloxacin in a pharmacodynamic model have demonstrated that these three agents possess equal bactericidal activity and are equally effective in preventing resistance development because the lower in vitro MICs for gatifloxacin and moxifloxacin were offset by the higher serum and tissue levels of levofloxacin (9). In the same study, ciprofloxacin did not exhibit rapid killing and selected for resistance faster than the other three agents (9). TRUST and other U.S. surveillance studies, using the NCCLS-recommended broth-dilution method, have shown that *S. pneumoniae* remain highly susceptible to levofloxacin with resistance rates in the United States of <1%; the $MIC_{90}$ for levofloxacin in these studies has remained at 1 mg/L from 1997 through 2002 (10–15). Further, levofloxacin, gatifloxacin, and moxifloxacin are equally effective in rates of clinical cure and microbiologic eradication of pneumococcal respiratory infections (16, and FDA website; available from: URL: http://www.fda.gov/cder/foi/nda/99/21061_Tequin.htm and http://www.fda.gov/cder/foi/nda/2001/21277_Avelox.htm)

The review implied that, in general, higher $AUC_{24h}$/MIC ratios were associated with better patient outcomes. For *S. pneumoniae*, several pharmacodynamic studies have demonstrated that a target $AUC_{24h}$/MIC ratio of 30 to 35 for fluoroquinolones is the best correlate for successful bacteriologic eradication, clinical cure, and prevention of emergence of resistance during therapy (5,9,17–19). Levofloxacin, gatifloxacin, and moxifloxacin all achieve this $AUC_{24h}$/MIC ratio (9). Zhanel et al. demonstrated that $AUC_{24h}$/MIC ratios above the target value of 30 to 35 did not improve bacteriologic eradication or reduce the emergence of resistance (9). Moreover, no clinical data support the claim that higher $AUC_{24h}$/MIC ratios correlate with better patient outcomes.

The review discusses the question of whether C-8-methoxyquinolones (moxifloxacin and gatifloxacin) have a lower propensity to select resistant mutants of *S. pneumoniae* compared with levofloxacin. Mutation prevention concentration is a theoretical laboratory concept based on agar dilution methodology, and no published data have shown any clinical correlation between this theory and clinical outcomes. NCCLS does not recommend agar dilution for susceptibility analysis of *S. pneumoniae*. Moreover, the extremely low levels of resistance in *S. pneumoniae* (<1%) after many years of fluoroquinolone use do not support the theory of mutation prevention concentration. The review did not reference an analysis of 16 penicillin-resistant *S. pneumoniae* strains by Kolhepp et al. (20). In that broth-dilution study, in vitro resistance developed in a greater proportion of strains exposed to gatifloxacin (11/16) and moxifloxacin (8/16) than to levofloxacin (2/16). Similarly, in a study by Klepser et al. that used an in vitro pharmacodynamic model, levofloxacin was less likely than moxifloxacin to select for resistant isolates

of *S. pneumoniae;* moreover, after 24 hours of exposure, levofloxacin MICs remained unchanged while moxifloxacin MICs increased two- to eightfold (21).

Levofloxacin, gatifloxacin, and moxifloxacin all have susceptibility rates >99% for *S. pneumoniae* (22,23). Although resistance is rare, considerable cross-resistance among fluoroquinolones is observed once two or more key mutations (e.g., $Ser^{79}$ in ParC, $Ser^{81}$ in GyrA) are detected (24,25). Using topoisomerase IV-selecting fluoroquinolones (ciprofloxacin and levofloxacin) in the same patient population as DNA gyrase-selecting fluoroquinolones (gatifloxacin and moxifloxacin) could potentially accelerate the development of double mutants (ParC and GyrA) and clinically important class resistance because selective pressure would be applied to both enzyme targets (26).

The review stated that, since 1999, at least 20 case reports of pulmonary infection that did not respond to levofloxacin therapy have been published. This number is remarkably small considering that >250 million patients have been treated with levofloxacin worldwide. A number of the treatment failures cited had documentation of prior ciprofloxacin use and ciprofloxacin failure, and many isolates were not tested for levofloxacin susceptibility before treatment (27). We agree with the recommendation in the cited Davidson et al. reference: a patient's failure to respond to one fluoroquinolone is sufficient reason not to use other fluoroquinolones (27). Isolated clinical failures will occur with the use of any antimicrobial agent when treating pneumococcal pneumonia.

The notion that fluoroquinolone therapy can be "targeted" for an indication requires challenge as fluoroquinolone therapy will always result in systemic drug levels. Evidence does not indicate that the use of two fluoroquinolones, such as ciprofloxacin and moxifloxacin, minimizes fluoroquinolone resistance. Targeted fluoroquinolone therapy may in fact have adverse implications for the patient and for overall institutional resistance patterns. For example, the use of ciprofloxacin for urinary tract infections exposes resident streptococci in the respiratory tract to an agent that has demonstrated weaker activity against pneumococci, thus potentially selecting for pneumococcal resistance (9). Moreover, 20%-35% of ciprofloxacin is excreted through the intestinal tract (Cipro package insert), compared to 4% of levofloxacin (Levaquin package insert). Studies have shown that ciprofloxacin displays weaker in vitro activity (lower percentage of isolates susceptible) than levofloxacin for several gram-negative enteric bacteria (2,3). Stepwise adaptive changes towards fluoroquinolone resistance in enteric bacteria may be selected by fluoroquinolones with weaker in vitro activity and higher levels of exposure in the intestinal tract. Therefore, ciprofloxacin would have a greater potential than levofloxacin for the selection of resistant strains of intestinal gram-negative pathogens. A recent report stated that ciprofloxacin-resistant *Escherichia coli* were isolated from the feces of 48% of patients treated with ciprofloxacin for prostatitis; before ciprofloxacin therapy, only ciprofloxacin-susceptible *E. coli* were isolated from the feces of these patients (28). Further, given that 25% of moxifloxacin is excreted through the intestinal tract (Avelox package insert), the use of moxifloxacin for respiratory infections exposes bacteria in the intestinal tract to a fluoroquinolone with greater activity against *Bacteroides fragilis* and other intestinal anaerobes than levofloxacin (29,30). Moxifloxacin has a greater potential than other fluoroquinolones to alter the normal intestinal flora and select for vancomycin-resistant enterococci (31) and intestinal gram-negative strains with increased fluoroquinolone resistance.

In conclusion, we believe that the data we have briefly presented here supplements the previous discussion by Scheld (1) and will help facilitate an improved understanding of the factors influencing the maintenance of fluoroquinolone efficacy.

Focus Technologies is the central testing laboratory for the TRUST antimicrobial susceptibility testing surveillance program, sponsored by Ortho-McNeil Pharmaceutical.

**Daniel F. Sahm,***
**Clyde Thornsberry,***
**Mark E. Jones,***
**and James A. Karlowsky***

*Focus Technologies, Herndon, Virginia, USA

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Address for correspondence: James A. Karlowsky, Focus Technologies, 13665 Dulles Technology Drive, Suite 200, Herndon, VA 20171-4603, USA; fax: (703) 480-2654; email: jkarlowsky@focusanswers.com

# International Travel and Sexually Transmitted Disease

**To the Editor**: Recent articles in the professional literature (1–3) have offered advice regarding the importance of taking a careful travel history, particularly in this time of unprecedented levels of international travel

(4). Such screening serves an important public health purpose as well, especially for sexually transmitted disease (STD) control.

Sexual behaviors associated with travel can change the level of risks for STD transmission (5–7), and the epidemiology of STDs is not uniform throughout the world (8,9). These geographic differences may increase the risk of a traveler's becoming infected, or, conversely, increase the risk of a traveler's introducing a sexually transmitted pathogen, possibly one that is resistant to treatment, into a low-incidence area (10). In addition, different strains of pathogens may be common in different parts of the world (11–14). For example, quinolone-resistant *Neisseria gonorrhoeae* (QRNG) is much more common in Asia (up to 40% of all isolates) (15). These strains of QRNG were first introduced in the United States by persons who engaged in sexual activity abroad, but now California and Hawaii have an increasing incidence of infection attributable to these strains (16). Indeed, QRNG has become endemic in those states, and incidence is no longer related to travel. During 1999–2001, only 3 QRNG isolates (0.28%) were identified among the 1,066 gonococcal isolates cultured in the STD Laboratory, State Laboratory Institute, Massachusetts Department of Public Health (Massachusetts Department of Public Health, unpub. data). However, in 2002, 9 (2.1%) of 425 isolates of *Neisseria gonorrhoeae* were quinolone resistant. None of the persons recently infected reported a history of travel outside of New England. Unfortunately, few had reliable information to identify their partner(s). Those partners who were identified were either not located or did not agree to speak with the disease intervention specialist.

This experience with antimicrobial resistance of *Neisseria gonorrhoeae* should serve as a model for STD prevention planning and programming. It highlights the importance of retaining the laboratory capacity to monitor antimicrobial susceptibilities of bacterial STD isolates. Treatment protocols should be adjusted in light of the prevalence of resistant strains of sexually transmitted pathogens. In cases in which symptoms associated with a bacterial STD persist after what is usually considered appropriate treatment, clinicians should obtain cultures and perform susceptibility tests on isolates. Nucleic acid amplification technologies do not provide critical antibiotic susceptibility information. In this situation, the public health STD program or laboratory should be contacted for guidance. Determining the sensitivity pattern of the pathogen in an expeditious fashion will ensure that appropriate and timely therapy can be initiated for the infected patient as well as enable more effective follow-up and treatment to sexual contacts. Asking patients who seek treatment for a possible STD about their own and their partner's travel histories is important to broaden the differential diagnosis (17). The increase in population mixing facilitated by travel and Internet-generated contacts may be diminishing the importance of the focality of traditional STD epidemiology. Finally, STD prevention messages should be a part of the health advice offered to travelers (7,18,19).

## Acknowledgments

We thank Alfred DeMaria and Ralph Timperi for their thoughtful reviews and comments regarding this material.

**Paul Etkind,* Sylvie Ratelle,* and Harvey George***

*Massachusetts Department of Public Health, Jamaica Plain, Massachusetts, USA

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Address for correspondence: Sylvie Ratelle, Bureau of Communicable Disease Control, Massachusetts Department of Public Health, 305 South Street, Jamaica Plain, MA 02130, USA; fax: 617-983-6925;. email: sylvie.ratelle.@state.ma.us

## *Salmonella* in Denmark

**To the Editor:** In the large study by Evans and Wegener recently published in Emerging Infectious Diseases (1), salmonellae in broiler chickens and pigs significantly decreased after routine in-feed antimicrobial drug use for growth promotion was terminated in Denmark. Avoparcin was a frequently used growth promoter in poultry until its ban in Denmark in 1995 because of its association with the development and spread of vancomycin-resistant enterococci. On examining Evans and Wegener's data, I noticed that a precipitous drop in salmonellae in broiler chickens appeared to have occurred in early 1996. Do the authors think this drop was due to the withdrawal of avoparcin? As the authors note, avoparcin has been associated with increased shedding of salmonellae (including a dose-response effect) in a number of studies (2,3). If the large drop (from approximately 25% positive samples in 1995 to approximately 10% in 1996) is not due to withdrawal of avoparcin, what do the authors suggest could have caused it?

Do the authors have sufficient numbers of samples to reanalyze their data in broiler chickens for three periods instead of just two (i.e., use the periods January 1995–December 1995, January 1996–December 1997, and January 1998–December 2000)? This change would take into account the potential effect of avoparcin withdrawal in 1995.

Also, the most important reason for decreasing food animals' carriage of salmonellae is to protect people from becoming ill with *Salmonella*. Do the authors have any figures on domestically acquired human infections with salmonellae in Denmark since early 1995? Is there any temporal association with the withdrawal of growth promoters?

**Peter Collignon***
*Sydney University, Woden, Australia

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Address for correspondence: Peter Collignon, Professor, Canberra Clinical School, Sydney University, P. O. Box 11, Woden, ACT. 2607, Australia; fax: 61 2 6281 0349; email: peter.collignon@act.gov.au

**In Reply:** The drop in *Salmonella* organisms in broiler chickens becomes evident in September 1995. The ban on avoparcin occurred in May 1995. These two facts suggest that the first flocks of broiler chickens produced without avoparcin were slaughtered in August 1995. Thus, the temporal relationship is evident. We have reanalyzed the data for the three strata January 1994–December 1995, January 1996–December 1997, and January 1998–December 2000. Each stratum is significantly different from the two others ($p < 0.0001$).

Arguing in favor of a causal relationship, apart from the temporal relationship, one would say that no changes in the Salmonella control program in this period could explain this reduction. Arguing against a causal relationship, one would say that the levels momentarily bounced back to nearly the pre-ban level in 1997, despite the avoparcin ban. The subsequent drop and consistent low level could be explained by changes in the control program (introduction of serologic *Salmonella* monitoring in 1997 to 1998). On the basis of our data, drawing a conclusion one or the other is not possible.

There is a clear temporal association between reduction in *Salmonella* in broiler chickens and reduced incidence of domestically acquired *Salmonella* infections that can be attributed to domestically produced broilers. This finding was recently reported in this journal (1).

**Mary E. Patrick***
**and Henrik C. Wegener†**
*DeKalb County Board of Health, Decatur, Georgia, USA; and †Danish Veterinary Institute, Copenhagen, Denmark

### Reference

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Address for correspondence: Mary Evans Patrick, DeKalb County Board of Health, 445 Winn Way, Decatur, GA 30030, USA; fax: 404-294-3842; email: mcevans@gdph.state.ga.us

# Industry-related Outbreak of Human Anthrax

**To the Editor**: In his letter, Industry-related Outbreak of Human Anthrax, Massachusetts, 1868 (1), Dr. Macher suggests an additional reference to cases of industry-related anthrax that occurred in the United States in the 1800s. Our paper exclusively addressed bioterrorism-related inhalational anthrax (2). In our introductory paragraph we referred to woolsorter's disease and ragpicker's disease, terms used to describe textile industry–related inhalational anthrax rather than cutaneous disease (3,4). The citation to which Dr. Macher refers describes eight patients that had cutaneous lesions consistent with cutaneous anthrax; some also had evidence of secondary bacteremic dissemination (5). Dr. Macher suggests that the symptoms of chest distress, chest pain, dyspnea, and tachypnea described in some of the patients are evidence of "mediastinal involvement." However, these symptoms may be consistent with bacteremic dissemination of *Bacillus anthracis,* and their presence is not sufficient evidence to conclude that these patients had hemorrhagic mediastinal lymphadenopathy, the pathologic hallmark of inhalational anthrax. In addition, the observed case-fatality rate of 25% in these patients is consistent with untreated cutaneous anthrax and contrasts sharply with the expected case-fatality rate of >85% for untreated inhalational disease (4,6).

**John A. Jernigan,***
**David S. Stephens,***
**David A. Ashford,***
**and Bradley A. Perkins***

*Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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Address for correspondence: John Jernigan, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS E68, Atlanta, GA 30338, USA; fax: 404-498-1244; email: jqj9@cdc.gov

**In Reply**: Jernigan et al. state that they exclusively addressed cases of inhalational anthrax in their 2001 report. However, I question whether their patient 8, a mail sorter with a "healing scab on the anterior neck," can be classically characterized as a pure case of inhalational anthrax.

On October 14, 2001, a 56-year-old female mail sorter (patient 8) in New Jersey became ill with vomiting and diarrhea, followed the next day by chills and fever. The vomiting and diarrhea improved, but during the next 2 days, she had shaking chills, fever, headache, and fatigue. A nonproductve cough developed, along with mild shortness of breath and anterior chest pain on inspiration. On October 19, with persistent fever and worsening chest pain, she went to a local emergency room. She appeared ill with increased respiratory effort and had a 0.5- to 1.0-cm healing scab on the anterior neck. A computed tomographic scan of the chest on October 22 demonstrated mediastinal and cervical lymphadenopathy (1).

In 1942, Severn reported a fatal case of pulmonary/meningeal anthrax in a 17-year-old woman whose cutaneous (point-of-entry) lesion had healed (2). The patient had received no specific treatment for anthrax, as such was not even suspected before death; nor had any form of chemotherapy been instituted. Up to 10 days before her illness, the woman had been working in a modern South Wales brush factory. Two days before ending her employment, she had what her parents described as a boil on the middle phalanx of the second finger of the right hand from the center of which her father extracted a bristle that had entered the finger in the course of her work. The lesion subsequently healed without medical aid. Her final illness (high temperature and severe headache) began 12 days after this incident, the patient being quite well in the interval.

An autopsy was performed by Dr. J. Gough, a pathologist at the Cardiff Royal Infirmary. Each pleural cavity contained blood-stained fluid: one half pint on the left side and three quarters pint on the right. The pia-arachnoid was extremely congested over the hemispheres, with subarachnoid hemorrhage on the upper and lateral aspects and along the Sylvian fissures. The brain showed an acute hemorrhagic inflammation of the pia-arachnoid over the hemispheres. The subjacent brain showed an acute inflammation of perivascular distribution in the gray matter. Similar inflammation was present in the basal ganglia and cerebellum. In the lungs, tissues were destroyed in some hemorrhagic areas. From the meninges, brain, spleen, and blood a spore-bearing aerobe was grown in pure culture. The same organism was grown from the lung. The spore-bearing organism was pathogenic for the guinea pig and mouse and identified as *Bacillus anthracis*. The woman had been

employed in sorting horsehair and pig bristle imported mainly from China and South America.

Samples of materials she sorted were tested for bacteria by Dr. V.D. Allison, Ministry of Health, who reported that the seven different batches of suspected horsehair and bristle submitted were heavily contaminated with aerobic spore-bearing organisms, and from one batch of mixed horsehair, he isolated a colony of typical *B. anthracis* that was lethal to a guinea pig.

The clinical history suggests that the portal of entry of the infection was the finger that healed spontaneously, as there was no evidence of this lesion at autopsy (2). I propose that Jernigan et al.'s patient 8 with inhalational anthrax may have also had a cutaneous portal-of-entry infection by the spores of *B. anthracis*.

Jernigan et al. also state that mediastinal involvement does not develop in patients with cutaneous anthrax. In 1918, Gilmour and Campbell (a pathologist) reported the cases of two men who contracted anthrax from shaving brushes contaminated with *B. anthracis*; patient 2 had cutaneous anthrax with mediastinal involvement. He was admitted to Bramshott Military Hospital, Canada, on February 27. On February 22, he had procured a new shaving brush from Quartermaster stores and used it for the first time on February 25; while shaving, he cut his left cheek slightly, causing free bleeding. Toward evening he began to feel poorly; he felt ill during the night. On the morning of February 26, he shaved again and reopened the wound, which again bled freely. Shortly before noon, his face began swelling very rapidly around the cut, down the side of his neck, and in front and behind the ear. During the afternoon he felt worse, had chills, and had a severe headache. He had difficulty swallowing, and the swelling of the neck and face was increasing. During the night, he had great difficulty in swallowing and breathing; he felt as if he were going to choke. He had a severe headache, nausea, vomiting, and chills. On February 27, his temperature was 38.9°C and pulse 140. The left side of his face and neck were very swollen; the swelling extended down over the sternum. Respirations were shallow and impaired. A smear from the malignant pustule on the left cheek demonstrated anthrax bacilli. On February 28, the patient lapsed into a coma and died. A postmortem examination showed that "mediastinal tissues were extremely edematous" (3).

Note the similarities between Gilmour and Campbell's patient 2 and Stone's patient 5, a laborer at a Massachusetts' animal hair factory (4). Patient 5 contacted Dr. Stone on November 17, 1867. He had been sick since the Thursday previous (14th) and had chills, pain in head and back, and loss of strength. He had previously noticed a pimple on his neck but could not say when it first appeared. The patient primarily had pain and distress in epigastrium and back. The pulse was 120, his breathing was hurried, and his neck was swollen. On November 18, the "slough" (cutaneous lesion) doubled in size, and on November 19, a severe chill developed and edema extended down to the nipple. On November 20, the patient's chest was doughy to the touch as far down as the nipples. On November 21, the patient became delirious, had chest pain, and died that evening. Stone's patient 5 may have also had mediastinal disease (5).

In conclusion, some persons who work in facilities that are contaminated with the spores of *B. anthracis* may experience dual cutaneous and inhalational anthrax infections, and mediastinal disease may develop in some patients with cutaneous anthrax.

**Abe Macher***

*Bethesda, Maryland, USA

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Address for correspondence: Abe Macher; Bethesda, MD, USA; fax: 301-443-8143; email: amacher@hrsa.gov

**In Reply:** Dr. Macher proposes that the cutaneous lesion on the neck of patient 8 in our series suggests a cutaneous entry. At the time of the patient's initial hospitalization for anthrax, the lesion and its history were evaluated with this possibility in mind. We do not believe the lesion was cutaneous anthrax. The lesion was present before the patient's exposure to *Bacillus anthracis* spores, and its clinical features and course were not compatible with cutaneous anthrax. The presence of mediastinal lymphadenopathy in this patient strongly indicates that the route of exposure was through inhalation.

Dr. Macher refers to case reports of patients with cutaneous anthrax who had evidence of secondary bacteremic dissemination. Patients with bacteremic cutaneous anthrax were also seen in the 2001 outbreak but were not included in our report. The findings in the patients referred to by Dr. Macher included edema, often extensive, of the skin and soft tissues contiguous to the primary cutaneous lesion, as well as edematous changes in multiple other organs and body spaces such as bowel wall, mesentery, omentum, central nervous system,

and peritoneal and pleural spaces (1–3). In one patient in whom the primary lesion was on the face, the postmortem findings included extensive edema of the neck, soft tissues of the chest, and the mediastinum (2). Mediastinal lymphadenopathy was not described in any of these patients. Edema is a well-described feature of *B. anthracis* infection believed to be the result of one of its two binary toxins, edema toxin, which likely causes edema by increasing cellular levels of cyclic AMP and upsetting water homeostasis (4).

Dr. Macher suggests that "mediastinal disease" may develop in cutaneous anthrax patients. We agree that cutaneous anthrax with bacteremic dissemination may result in pathologic changes in multiple organs. Mediastinal edema can result from hematogenous infection of mediastinal tissues, by direct extension from the involved structures of the face and neck, or from systemic effects of circulating toxin. However, edema should not be confused with hemorrhagic mediastinal lymphadenopathy, the classic pathologic change associated with inhalation anthrax. We are unaware of any evidence, including that presented in the case reports referenced to by Dr. Macher, suggesting that hemorrhagic mediastinal lymphadenopathy results from cutaneous anthrax infections. This signature finding in inhalational anthrax is thought to result from phagocytosis of inhaled spores by alveolar macrophages, followed by transportation within phagocytes to the mediastinal lymph nodes where initial multiplication of the bacilli and release of toxin occurs (4).

**John A. Jernigan,***
**Martin S. Topiel,†**
**and David S. Stephens***

*National Center for Infectious Diseases Centers for Disease Control and Prevention, Atlanta, Georgia, USA; and †Virtua Health, Mount Holly, New Jersey, USA

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Address for correspondence: John Jernigan, Centers for Disease Control and Prevention, 1600 Clifton Rd., MS E68, Atlanta, GA 30333; fax: 404-498-1244; email: jqj9@cdc.gov

**Correction, Vol. 9, No. 9**

On p. 1326, in the article on "Mayaro Virus in Wild Mammals, French Guiana," in the 8th line under "The Study," a serum was incorrectly printed as a dilution. The line should read as follows: Serum samples with titers >20 were confirmed by seroneutralization at a 1:20 dilution (10).

## Open Access Publishing Conference

An Open Access Publishing Conference will be held on January 7, 2004 at Emory's Woodruff Health Sciences Center Administration Building Auditorium. The keynote address will be delivered by Dr. Harold Varmus, early advocate of a new model for disseminating scientific research. Other speakers include representatives from the National Library of Medicine, BioMed Central, and the library community. This half-day conference, which is jointly sponsored by CDC Information Center & Emory Health Sciences Center Library, is open to all interested faculty, scientists, public health workers, and librarians. Registration is required. For more information, contact the CDC Information Center at 404-639-1717.

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# Emerging Infectious Diseases: Trends and Issues

**F.R. Lashley and J.D. Durham, editors**

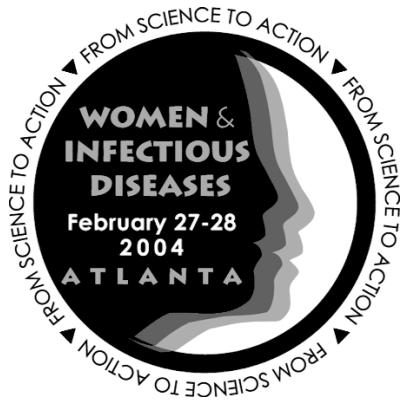
**Springer Publishing Company, New York, New York, 2002, ISBN: 0-8261-1474-1, Pages: 483, Price: $58.95**

Preparing a text about emerging and reemerging infections sounds like a contradiction in terms since by the time a book is published, "new" infections may have come and gone. But Lashley and Durham have successfully walked the thin line between being dated on the one hand and providing timely, relevant data on the other. Several chapters place emerging infections and related problems such as microbial resistance in historical, cultural, and environmental context, which is relevant across diseases and time. The case study approach used for 17 specific diseases (e.g. cholera, cryptosporidiosis, malaria, prions, drug-resistant *Streptococcus pneumoniae*, West Nile virus) makes for an excellent vehicle for learning and fascinating reading. The book has five chapters on special issues—the role of infections in some cancers and chronic diseases, travel, immunocompromised persons, bioterrorism, behavioral and cultural aspects of transmission and infection—which cut across disease categories, as well as a future-looking summary. The book has four appendices for quick reference: emerging infections by organism and mode of transmission, prevention strategies, and a thorough list of resources. This book can serve as a valuable resource for epidemiologists, graduate students, and clinicians who need an overview reference text.

**Elaine Larson***

*Columbia University, New York, New York, USA

Address for correspondence: Elaine Larson, Columbia University School of Nursing, 630 W. 168th St., New York, NY 10032, USA; fax: 212-305-0722; email: ell23@columbia.edu




## International Conference on Women and Infectious Diseases: from Science to Action

The International Conference on Women's Health and Infectious Diseases, sponsored by the Centers for Disease Control and Prevention (CDC) and partners, will be held at the Marriott Marquis, Atlanta, Georgia, February 27–28, 2004. Intended for clinicians, scientists, women's health advocates, health educators, public health workers, academicians, and representatives from all levels of government and from community-based, nonprofit, philanthropic, and international organizations, the conference will promote prevention and control of infectious diseases among women worldwide.

Featured sessions will include women and HIV/AIDS, perinatal infectious diseases, immunizations, links between infectious and chronic diseases, and the impact of globalization. Other topics include infectious disease disparities, gender-appropriate interventions, and effective health communication.

Speakers will include, Julie L. Gerberding, CDC director, who will speak about the impact of infectious diseases on women; Carol Bellamy, executive director, United Nations Children's Fund (UNICEF), who will speak about globalization and its effect on infectious diseases among women; and Mirta Roses Periago, director, Pan American Health Organization (PAHO), will speak about prevention of infectious diseases among women globally.

**For information, about cost and registration, contact the Office of Minority and Women's Health, National Center for Infectious Diseases, CDC, at Web site: www.womenshealthconf.org; email: omwh@cdc.gov; or phone: BeJaye Roberts, 404-371-5492.**

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Contact: 703-535-6862
email: info@retroconference.org
Web site: www.retroconference.org

**February 27–28, 2004**
International Conference on Women and Infectious Diseases: From Science to Action
Atlanta Marriott Marquis
Atlanta, GA
Contact: BeJaye Roberts, 404-371-5492
Web site:
http://www.womenshealthconf.org

**February 23–25, 2004**
International Conference on Animal Welfare
Office International des Epizooties
Paris, France
Contact: 33 (0)1 44 15 18 88
email: oie@oie.int
Web site: www.oie.int

**Michelangelo Merisi da Caravaggio (1571–1610). Basket of Fruit (1596)**
Oil on canvas, 45.92 cm x 64.46 cm Pinacoteca Ambrosiana, Milan, Italy

Born Michelangelo Merisi, Caravaggio was later renamed after his hometown in northern Italy, a practice not unusual in his day. His father, an architect and majordomo to the Marquis of Caravaggio, died of the plague when the artist was still young, leaving him under the protection of the art-loving marquis. Like many children of his day, he learned early how to grind pigments for painting, and soon he was apprenticed to a good studio in Milan. At 21, he moved to Rome, anxious, if not fully qualified, to compete in the capital's bustling art world. This move to Rome began the tumultuous life journey of a man who changed the art of his day, had many followers (the Caravaggisti), and influenced future masters, from Rembrandt to Velázquez (1).

In Rome's cosmopolitan art scene, the young Caravaggio found scant opportunity and slow recognition. Handicapped by his exuberance, fiery temper, and heightened artistic sensitivity, he was unable to cope with restrictions and authority. Brash, overbearing, and irascible, he became entangled in riotous brawls and walked the disorderly side of the capital. All the while, he painted mellow canvases overflowing with empathy, humanity, and compassion. Inventing a new, radical kind of realism, he populated his pictures with ordinary people, embracing their imperfections and weaknesses with a candor that many of his contemporaries mistook for vulgarity (2).

As if to decipher the contradictions and paradoxes of his own shadowy character, Caravaggio explored the interplay of light and dark, known in Italian as chiaroscuro. In an exaggerated theatrical style, he cast light selectively, adding drama to scenes, illuminating figures, and creating a poetic reality that was both earthy and mystical (3).

With remarkable immediacy, he painted potent images of beheadings and executions, perhaps anticipating the horror of his own punishment for unsavory behavior, not the least of which was killing his opponent during a tennis game. Arrested, imprisoned, pardoned, and constantly on the edge, Caravaggio continued to paint while living in exile for 4 years. The disregard for limits that distinguished his work dominated his life and in the end overcame his artistic promise. Injured during one final, ironically mistaken, arrest, and feverish with malaria, he died before age 40 (4).

During the early days of his tenure in Rome, unknown, unemployed, and unappreciated, Caravaggio painted religious images and baskets of fruit and flowers. Still-life painting, the domain of beginners since antiquity, ranked low on the hierarchical order of pictorial genres. Reduced to it by circumstance, Caravaggio elevated the genre to new heights, creating a European tradition that explored the "secret lives of objects" (5).

"I put as much effort in painting a basket of flowers as I do in painting human figures," Caravaggio told an early patron (3). In an innovative move toward abstraction, he allowed objects (their form, angle, solidity, composition) to define space. Instead of idealizing them, as the classicists advocated, he painted their imperfections, investing them with uniqueness and content. And instead of centering compositions on the canvas, he thrust them provocatively in the viewer's face, demanding attention and participation.

"I would have…hung a similar basket next to it but as no one was able to attain its incomparable beauty and excellence, it remained alone," 17th-century cardinal Federico Borromeo said of Caravaggio's Basket of Fruit (6), on this month's cover of Emerging Infectious Diseases.

This "incomparable" basket, probably painted over a number of days, has a weathered familiarity, its ripened contents settled, its branches jutting stiffly out the edge. Though representing tradition and plentitude, the fruit is past its prime. Only the tart quince seems to be holding firm. Soft and lusterless, the apple is pockmarked and flawed. The grapes hang heavy, their translucent skin spotted and brown against the plump figs. The leaves, colors fading, edges curling and snarled, are brittle and crinkly. Yet, against an abstract backdrop of brilliant gold leaf, this laden basket exudes comfortable elegance, tangible beauty, graceful maturity.

Caravaggio's painting is not just a lyrical composition of forms. Engaging the senses in virtual abundance, which like life itself is all too ephemeral, the basket comments

on the complexity and vanity of nature. Defying the moment of creation, the diverse image spans instead the life of the fruit, reflecting on its inevitable decay. The blemishes, intentional and central to the theme, are not brought on by precipitous mishap but by nature. Uncontrolled environment (temperature, moisture, microorganisms) has disrupted the fruit's normal physiology, devitalizing the skin, allowing invasion of pathogens, and promoting decomposition.

In our world, as in Caravaggio's, where light and darkness, beauty and horror, engagement and danger are constantly at play, survival depends on keeping the elements of nature in balance, constantly tracking their course, monitoring their moves, and checking their excesses. Left untended and uncontrolled, nature's elements will thrive to unfair advantage, mutate to our detriment, and travel to our doorstep. In Mongolia, Vietnam, and other formerly out-of-the-way places, where control efforts have not always kept pace, old scourges (tuberculosis, brucellosis, plague, tularemia) maintain their insidious hold, a blemish on world health and a threat to balance and control.

**Polyxeni Potter**


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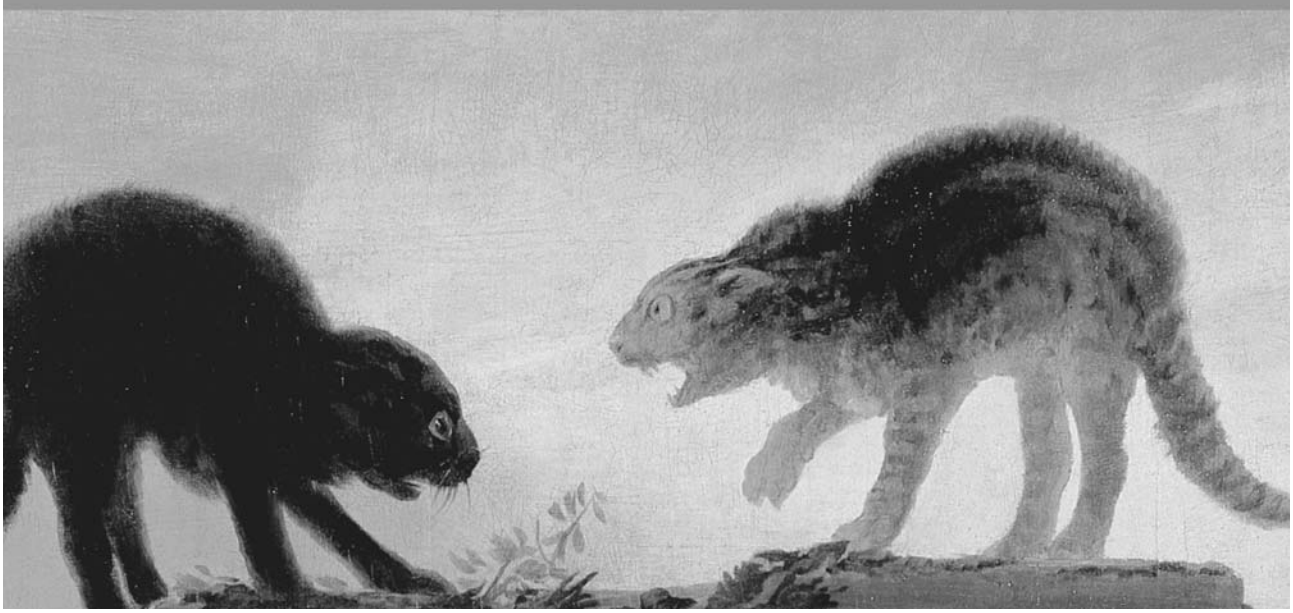
5. Wheelock AK (Jr). Still lifes of the golden age: northern European paintings from the Heinz family collection. Catalog entries by Ingvar Bergstrom. Washington, D.C.: The National Gallery of Art; 1989.
6. Abrosiana Gallery—interesting facts [cited 2003 Oct]. Available from: URL: http://www.rcs.it/mimu/english/musei/pinacoteca_ambrosiana/curiosita.htm



EMERGING INFECTIOUS DISEASES

A Peer-Reviewed Journal Tracking and Analyzing Disease Trends Vol.10, No.1, January, 2004

# Upcoming Issue

**For a complete list of articles included in the January issue, and for articles published online ahead of print publication, see http://www.cdc.gov/ncidod/eid/upcoming.htm**

## Look in the January issue for the following topics:

*Salmonella enteritidis* Infections, United States, 1985-1999

Emerging Issues in Virus Taxonomy

Severe Acute Respiratory Syndrome-associated Coronavirus in Lung Tissue

Ecologic and Geographic Distribution of Filovirus Disease

Fatal-Infection Surveillance and the Medical Examiner Database

Hospital-reported Pneumococcal Susceptibility to Penicillin

Ciprofloxacin-resistant *Salmonella enterica* Typhimurium and Choleraesuis from Pigs to Humans, Taiwan

*Escherichia coli* Producing CTX-M-2 ß-Lactamase in Cattle, Japan

Respiratory and Urinary Tract Infections, Arthritis, and Asthma Associated with HTLV-II and HTLV-I Infection

Fluoroquinolones Protective against Third-Generation Cephalosporin Resistance in Gram-negative Nosocomial Pathogens

*Bacillus anthracis* Incident, Kameido, Tokyo, 1993

EMERGING INFECTIOUS DISEASES www.cdc.gov/eid

# JOURNAL BACKGROUND AND GOALS

### What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

### Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

### What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

## 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 and Historical Reviews, Dispatches, Commentaries, Another Dimension, Letters, Book Reviews, and News and Notes. 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 home page at http://www.cdc.gov/eid.

## Instructions to Authors

**Manuscript Preparation.** For word processing, use MS Word. Begin each of the following sections on a new page and in this order: title page, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

**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). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and e-mail address). Include separate word counts for abstract and text.

**Keywords.** Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

**Text.** Double-space everything, including the title page, abstract, references, tables, and figure legends. Printed manuscript should be single-sided, beginning with the title page. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

**Biographical Sketch.** Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

**References.** Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not 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. List the first six authors followed by "et al." Do not cite references in the abstract.

**Tables and Figures.** Create tables within MS Word's table tool. Do not format tables as columns or tabs. Send graphics in native, high-resolution (200 dpi minimum) .TIF (Tagged Image File), or .EPS (Encapsulated Postscript) format. Graphics should be in a separate electronic file from the text file. For graphic files, use Arial font. Convert Macintosh files into the suggested PC format. Figures, symbols, letters, and numbers should be large enough to remain legible when reduced. Place figure keys within the figure. For more information see EID Style Guide (http://www.cdc.gov/ncidod/EID/style_guide.htm).

**Manuscript Submission.** Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch) and verifying that the final manuscript has been seen and approved by all authors. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases website (www.cdc.gov/eid).

## Manuscript Types

**Perspectives.** Articles should be under 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 of first author. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and 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.** Articles should be under 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 of first author—both authors if only two. 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Research Studies.** Articles should be under 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 of first author—both authors if only two. 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 (i.e., "Here is what we found, and here is what the findings mean").

**Policy and Historical Reviews.** Articles should be under 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. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

**Dispatches.** Articles should be 1,000–1,500 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed two); and a brief biographical sketch of first author—both authors if only two. Dispatches 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.

**Commentaries.** Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but should not include figures or tables.

**Another Dimension.** Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit.

**Letters.** This section includes letters that present preliminary data or comment on published articles. Letters (500–1,000 words) should not be divided into sections, nor should they contain figures or tables. References (not more than 10) may be included.

**Book Reviews.** Short reviews (250–500 words) of recently published books on emerging disease issues are welcome. The name of the book, publisher, and number of pages should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted on the journal Web page only, depending on the event date.)

**Conference Summaries.** (500–1,000 words) of emerging infectious disease conferences may provide references to a full report of conference activities and should focus on the meeting's content rather than on individual conference participants.