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March 2010

Vector-borne Infections

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Emerging Infectious Diseases
Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

EMERGING INFECTIOUS DISEASES

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On the Cover

William Blake (1757–1827)
The Ghost of a Flea (1819–20)
Tempera mixture panel with gold on mahogany
(21.4 cm × 16.2 cm)
Tate, London, England

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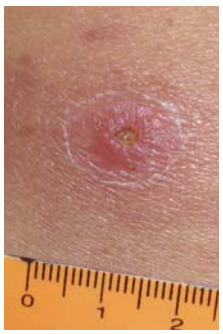

Another Dimension

Letters

About the Cover

Preparing a Community Hospital to Manage Work-related Exposures to Infectious Agents in BioSafety Level 3 and 4 Laboratories

George F. Risi, Marshall E. Bloom, Nancy P. Hoe, Thomas Arminio, Paul Carlson, Tamara Powers, Heinz Feldmann, and Deborah Wilson

Construction of new BioSafety Level (BSL) 3 and 4 laboratories has raised concerns regarding provision of care to exposed workers because of healthcare worker (HCW) unfamiliarity with precautions required. When the National Institutes of Health began construction of a new BSL-4 laboratory in Hamilton, Montana, USA, in 2005, they contracted with St. Patrick Hospital in Missoula, Montana, for care of those exposed. A care and isolation unit is described. We developed a training program for HCWs that emphasized the optimal use of barrier precautions and used pathogen-specific modules and simulations with mannequins and fluorescent liquids that represented infectious body fluids. The facility and training led to increased willingness among HCWs to care for patients with all types of communicable diseases. This model may be useful for other hospitals, whether they support a BSL-4 facility, are in the proximity of a BSL-3 facility, or are interested in upgrading their facilities to prepare for exotic and novel infectious diseases.

Over the past decade, biomedical research performed on agents of viral hemorrhagic fevers (VHFs) has substantially increased. These agents are members of several virus groups, including filoviruses (Ebola virus, Marburg virus), Old World arenaviruses (Lassa virus, Lujo virus), New World arenaviruses (Machupo virus, Junin virus, Sabia virus, Guanarito virus, Chapare virus), flaviviruses (Omsk hemorrhagic fever virus, Kyasanur Forest disease virus), and bunyaviruses (Crimean–Congo hemorrhagic fever virus, Rift Valley fever virus) (*1*). Work with these agents is performed in specialized containment laboratories, operating at either BioSafety Level (BSL) 3 or BSL-4. BSL-3 denotes the potential for aerosol transmission to the laboratory worker. An agent that also is associated with high lethality and for which no available vaccine or specific treatment exists is studied at BSL-4 (*2*). Many VHF agents have a demonstrated potential for person-to-person transmission, including in nosocomial settings. A recent example of person-to-person transmission to hospital personnel occurred in September and October 2008 when Lujo virus was transmitted from the index patient to a paramedic, 2 nurses, and a member of the janitorial staff. Barrier precautions were not in place at the time of these events (*3*).

To provide safe work settings in which to study these pathogens, several BSL-4 laboratories are either in operation or under construction in the United States and abroad (Table 1) (T.G. Ksiazek, pers. comm.). Operation and management of these facilities are characterized by redundant engineering of safety features, strict administrative oversight, biosecurity measures, and extensive training (*2*,*4*), all designed to reduce the risk for exposure to persons working in this environment and prevent agents from being released into the community. Despite these safeguards, researchers in the United States and abroad have, on occasion, sustained occupational exposures to such agents, which rarely have resulted in overt illness and death (Table 2) (*5*–*11*). Because of the potential for person-to-person transmission of many VHF agents, rendering care to exposed or ill persons requires considerations beyond the scope of traditional hospital practices. Contact and/or airborne isolation guidelines may need to be added to standard isolation over the course of a patient's hospitalization (*12*,*13*).

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DOI: 10.3201/eid1603.091485

Table 1. BSL-4 laboratories planned or operational, 2009*

Location	Status
United States	
Centers for Disease Control and Prevention, Atlanta, GA, USA	A
Georgia State University Viral Immunology Center, Atlanta	A
Boston University National Emerging Infectious Disease Laboratories, Boston, MA, USA	NA
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA	A
Department of Homeland Security National Biodefense Analysis and Countermeasures Center, Frederick, MD, USA	NA
National Institute of Allergy and Infectious Diseases	
Integrated Research Facility, Frederick	NA
Rocky Mountain Laboratories, Hamilton, MT, USA	A
Southwest Foundation for Biomedical Research, San Antonio, TX, USA	A
University of Texas Medical Branch, Galveston, TX, USA	
Robert E. Shope MD BSL-4 Laboratory	A
Galveston National Biocontainment Laboratory	A
Other countries	
Geelong, Victoria, Australia	A
Winnipeg, Ontario, Canada	A
Taiwan	NA
London and Salisbury, UK	A, A
Lyon, France	A
Libreville, Gabon	A
Hamburg, Marburg, Berlin, and Greifswald, Germany	A, A, A, NA
Pune, India	NA
Rome, Italy	A
Bilthoven, the Netherlands	A
Novosibirsk, Russia	A
Sandringham, South Africa	NA
Solna, Sweden	A
Geneva and Spiez, Switzerland	A, NA

*BSL-4, BioSafety Level 4; A, active; NA, nonactive.

On several occasions, persons naturally infected with a VHF agent have sought treatment at hospitals located in industrialized areas of the world (*14*–*21*). Often the correct diagnosis is not considered at the time of hospitalization, and only standard isolation is used until such time as the diagnosis is suspected or confirmed. Despite this limitation, nosocomial transmission of these agents is uncommon in adequately resourced hospitals (*16*,*18*,*20*,*21*). Notably, the medical care requirements for patients with a naturally acquired VHF illness are identical to those needed for laboratory-acquired infections with the same agents.

Because of the limited and unique settings in which BSL-4 research has historically taken place in the United States, hospitalization for occupational exposures to VHF agents has typically been a dedicated facility remote from a conventional hospital, e.g., the medical containment suite (the "slammer") at the US Army Medical Research Institute of Infectious Diseases (USAMRIID), Frederick, Maryland, USA, or the biocontainment patient care unit at Emory University, Atlanta, Georgia, USA. The benefits of a remote facility include reducing the risk for nosocomial transmission, use of personnel who are already trained in managing a patient in containment, and control of public access (*22*). However, this approach has several serious drawbacks, including limited access to medical specialties and nursing staff, limited availability of medications and blood products, and limited access to specialized equipment such as ventilators and hemodialysis machines. In addition, increased psychological stress is experienced by patients confined to such a facility. Finally, given that the need to activate these facilities is extremely rare, the expense of building and maintaining a stand-alone unit poses a substantial limitation to this approach.

In addition to physical separation of the facility, medical and support staff at the USAMRIID facility work in positive pressure suits similar to those used in the laboratories themselves (*22*). Although the use of such suits provides protection to the caregiver, positive pressure suits are cumbersome, physically demanding to work in, and require substantial time for donning and doffing (dressing and undressing). Furthermore, venipuncture and other interventions in this unaccustomed and inconvenient setting pose a clear exposure risk to healthcare workers (HCWs). These factors are serious drawbacks when a HCW needs to render care to an acutely ill patient.

Documented clinical experience from several situations clearly indicates that nosocomial transmission can be prevented by implementing standard, contact, and airborne isolation procedures (*3*,*15*,*16*,*19*,,*20*). Furthermore, all BSL-4 research programs stress the importance of recognizing and quickly reporting potential work-related exposures and illnesses to occupational medical and safety staff. Thus, healthcare staff will typically be informed about the specific agent and the nature of the exposure early in the incubation period. This will enable rapid evaluation and timely institution of appropriate isolation precautions.

Given all these considerations, what additional enhancements are really necessary for a hospital to safely care for patients while still enabling delivery of optimum medical care? Because of sensational misconceptions about VHF agents in popular media such as movies and the press, other serious issues are the willingness of HCWs to render care to such persons and how to determine what additional actions would increase the likelihood of their doing so. We offer a practical approach to dealing with these issues in the procedures followed by a patient isolation facility located in Missoula, Montana, USA, and its attendant training and educational components.

Table 2. Infections caused by laboratory exposure to hemorrhagic fever viruses*

Virus	Incident
Ebola	Fingerstick while manipulating infected guinea pig tissue, 1977 (*5*); percutaneous exposure to blood from a Zaire Ebola virus–infected rodent, 2004 (*7*)
Marburg	3 laboratory acquired infections since the mid-1980s; 1 death occurred in Russia; no details available (*8*)
Crimean–Congo hemorrhagic fever	8 cases before 1980 compiled by SALS; no details available (*9*)
Lassa	1 case reported in 1970 with limited details provided (*10*)
Junin	21 cases before 1980 compiled by SALS; no details available (*9*)
Machupo	1 person exposed to aerosolized blood from a broken test tube (*11*)

*SALS, Subcommittee on Arbovirus Laboratory Safety.

Care and Isolation Unit

The Division of Intramural Research of the National Institute of Allergy and Infectious Diseases (NIAID) recently completed construction of an integrated research facility with BSL-4 research space at its Rocky Mountain Laboratories (RML) in Hamilton, Montana. As part of the project, NIAID contracted with St. Patrick Hospital and Health Sciences Center (SPH), a regional referral medical center located in Missoula, Montana, for provision and staffing of a patient isolation facility to support the RML BSL-4 research program. The facility, known as a care and isolation unit (CIU) (*23*) was designed to care for RML workers who had either known or had potential exposure to, or illness from, work-related diseases. The facility had to be located within 75 miles of RML, had to provide the full range of standard in-patient care, including intensive care, and had to meet the facility design guidelines of the National Institutes of Health, Division of Occupational Health and Safety (NIH DOHS) (*24*). Furthermore, the hospital had to supply the personnel to provide the full range of medical and nursing care and to be able to accept a patient within 8 hours (this would entail notification of key members of the hospital hierarchy, transferring patients if the rooms were currently occupied, securing adequate nursing and support staff, and carrying out systems checks to ensure that air handling systems and autoclaves were operational). In addition to the physical facility, a training program for critical care nurses, physicians, and other medical personnel was a major component of the contract.

To satisfy the NIH requirements for the CIU, the following elements were needed: 1) access control, i.e., the ability to restrict entrance into the CIU to authorized persons only; 2) three separate stand-alone rooms, each with a bathroom and shower, separate air handling, and an anteroom separating the patient room from the hallway; 3) directional air flow from the hallway into the anteroom and from the anteroom into the patient room; 4) a dedicated exhaust system providing ≥12 air exchanges per hour to the patient rooms (including ≥2 outside air changes per hour); 5) passage of exhaust through a HEPA filter to the building exterior ≥8 feet above the rooftop and well removed from air intake ducts; 6) room surfaces constructed of seamless materials amenable to topical disinfection; 7) the capability for the full range of intensive care unit (ICU) monitoring and support, including the ability to perform limited surgery, hemodialysis or peritoneal dialysis, Swan-Ganz catheter placement, and hemodynamic monitoring; and 8) a separate autoclave within the CIU for sterilizing all items that come out of a patient room.

SPH was selected to provide these services and facilities. SPH is a not-for-profit medical center under the sponsorship of the Sisters of Providence. It has 195 acute care beds, and >10,000 patient admissions per year. The full range of standard specialty medical care is available within the hospital, including 24 hour, 7 day/week availability of specialists in critical care, infectious disease, and all surgical subspecialties.

SPH retrofitted 3 adjacent rooms within the existing medical ICU (MICU) to create the CIU. A set of doors was installed to control access to the CIU from the MICU, and these would remain open when the CIU was not in use (Figure). A separate fully equipped nursing station was constructed, with closed circuit television monitoring for each of the 3 rooms. After construction, the CIU was inspected and

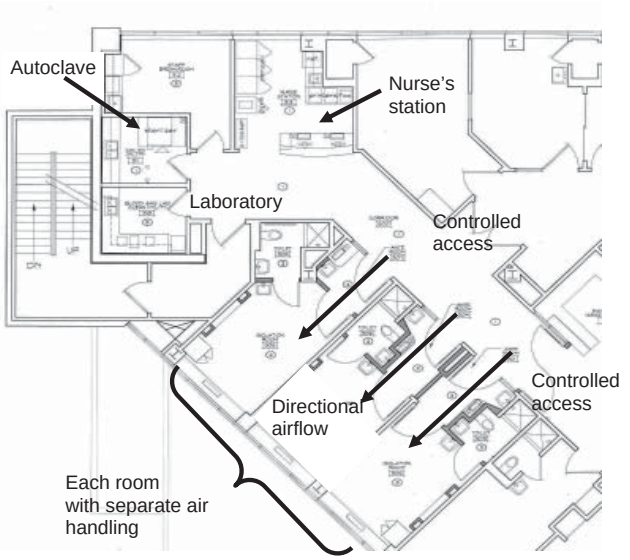

Figure. Floor plan of the Care and Isolation Unit, St. Patrick Hospital and Health Sciences Center, Missoula, MT, USA.

approved by officials from NIH DOHS. Under normal circumstances, the CIU operates either as 3 conventional MICU rooms or as isolation rooms for patients with community-acquired illnesses for which isolation of airborne pathogens is needed. If a patient from RML should require admission, any current occupants would be transferred, and access would be limited by closing off that section of the MICU.

In addition to the physical aspects of the CIU, several other elements were developed. Specific policies and procedures were written that deal with all aspects from admission to discharge, including unique aspects such as clean up of infected bodily spills, donning and doffing of personal protective equipment (PPE), and use of the autoclave. Support of hospital administration, physicians, nurses, and support personnel was critical. This backing was enlisted primarily by mounting an educational campaign that stressed the true risk for nosocomial transmission of these agents, as well as the recognition that the increased resources that would be provided to the hospital could greatly enhance capacity for handling community-acquired infections.

One feature dealt with preparing the hospital staff to care for such exposed persons. To accomplish this feature, we developed a detailed curriculum, which can be presented during a 1-day training workshop. This workshop includes didactic information, patient care scenarios discussed in group settings, and hands-on training. Simulation of various patient care activities (hand hygiene, donning and doffing of PPE, cleanup of body fluids, and rendering ICU level care to a patient) is conducted by using programmable mannequins and either tonic water or Glo Germ (Glo Germ, Moab, UT, USA), both of which fluoresce under ultraviolet light, to simulate infectious body fluids. Continuing education credits are granted for participation. Competence is maintained with quarterly demonstration of proper technique, review of CIU-specific policies and procedures, and required utilization of a series of online problem-oriented patient care scenarios. Training videos have been developed that demonstrate proper technique for spill cleanup, donning and doffing of PPE, processing of patient specimens, and processing of biohazardous waste, including use of the autoclave. Finally, detailed educational modules have been developed for each of the BSL-4 pathogens. These modules are designed to provide a nurse, emergency medical technician, or critical care physician with critical information that is quickly accessible as well as an extensive discussion of all aspects of the agent. The modules are in a standard format with extensive references and websites for further reading. All of this information is available for review any time both in hard copy as well as on the hospital's intranet site in the form of slide presentations, videos, or PDF files. The SPH staff has been generous in supplying feedback on the training and has been instrumental in refining the curriculum. Acquisition of knowledge has been documented with the use of pretesting and posttesting. After completion of the training, SPH staff members expressed increased confidence in caring for patients with all types of communicable infectious diseases, including VHFs.

To maintain readiness, a series of drills and exercises have been performed and will continue, in collaboration with RML and local emergency medical services providers. These readiness exercises have encompassed all aspects of care from arrival to the hospital through discharge.

Discussion

Engineering and administrative controls as well as PPE and standard operating procedures that are in place in modern BSL-4 laboratories have been associated with a greatly reduced incidence of occupational exposures to infectious agents (*23*,*25*). However, exposures, now primarily by the percutaneous route, still occur. USAMRIID recently published a review of potential laboratory exposures to agents of bioterrorism at their facility during 1989–2002 (*26*). During that time, 12 evaluations were made for potential exposures to filoviruses (Ebola virus or Marburg virus), 3 to arenaviruses, and 4 to Crimean–Congo hemorrhagic fever virus. Although none of these incidents was deemed a high enough risk to warrant isolation of the exposed persons, 2 laboratory workers were given investigational antiviral agents. One exposure at USAMRIID in 2004 resulted in isolation when a scientist received a puncture injury through a gloved hand while manipulating a mouse that had been experimentally exposed to Ebola virus (*22*). Fortunately, none of these situations resulted in infection. However, workers have been infected by agents of VHF from laboratory accidents elsewhere (Table 2).

Nosocomial transmission of VHF is infrequently described outside of resource-poor settings. With rare exception, such events have occurred because of the lack of recognition that the index patient had such an infection (*3*,*18*). The Centers for Disease Control and Prevention (CDC) has published guidelines for management of patients infected with viral hemorrhagic fevers in the conventional hospital setting (*12*,*13*). Notably, medical care has been safely rendered by using conventional barrier precautions alone to persons infected with VHF viruses, including Ebola virus (*5*,*18*), Marburg virus (*19*,*20*), Lassa fever virus (*27*), Machupo virus (*11*), Sabia virus (*28*), and Crimean–Congo hemorrhagic fever virus (*21*).

Nevertheless, even well-trained HCWs may make mistakes due to anxiety, fatigue, or other stressors, so additional facility enhancements that augment safety are desirable when dealing with potentially lethal infectious diseases. Furthermore, the recognition of a patient with an exotic or unfamiliar contagious disease may engender trepidation among the medical community as well as the public. Such concerns have at times resulted in reluctance on the part

of HCWs to care for persons infected with such agents as monkeypox virus (*29*), *Yersinia pestis* (plague) (*30*), and others. When a sample of 1,000 physicians were surveyed (526 responses), 80% indicated a willingness to care for patients in the event of an outbreak of an unknown but potentially deadly illness, but only 21% felt adequately prepared to do so (*31*). Reluctance is often out of proportion to the true risks and results from concerns for personal and family member safety. These concerns are likely to be reduced if the HCW perceives that the facility has taken additional precautions and instituted additional training.

To maximize safety as well as to address provider concerns of HCWs and other staff, we have developed the CIU and our accompanying training program. Our pragmatic and practical approach provides a well-designed facility that enhances safety not only for the care of a patient infected with a laboratory-acquired VHF virus infection, but also for serious transmissible community-acquired disease or for exotic diseases contracted while traveling.

As international tourism and work assignments continue to expand, the importation of exotic diseases is almost certain to increase and to appear in unexpected locations. Recent instances of infection have occurred with Marburg virus in Colorado (*19*) and the Netherlands (*20*); with Lassa fever virus in New Jersey (*15*), the United Kingdom (*16*), and Germany 6 (*16*); with *Y. pestis* (*32*) in New York, New York; and with (initially thought) extensively drug-resistant *Mycobacterium tuberculosis* in Atlanta, Georgia (*33*). Finally, in the United States, 1,356 BSL-3 laboratories are registered with either CDC or the US Department of Agriculture select agent programs (*34*). For these reasons, relatively low-cost facilities ($624,000.00 for design and construction of our unit) like the CIU may become more critical. Furthermore, training programs, similar to the one we have implemented, with emphasis on such practical infection control issues as the proper use of PPE, hand hygiene, and proper spill cleanup, has broad application. Other communities might consider the benefits of our approach, whether or not infectious disease research laboratories are constructed in their area.

This work was supported by the NIH Division of Occupational Health and Safety (G.R., N.H., T.A., P.C., D.W.), and the NIAID Division of Intramural Research (M.B., H.F.).

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Bartonella spp. Transmission by Ticks Not Established

Sam R. Telford III and Gary P. Wormser

Bartonella spp. infect humans and many animal species. Mainly because PCR studies have demonstrated *Bartonella* DNA in ticks, some healthcare providers believe that these microorganisms are transmitted by ticks. *B. henselae,* in particular, is regarded as being present in and transmissible by the *Ixodes scapularis* tick. The presence of a microbial agent within a tick, however, does not imply that the tick might transmit it during the course of blood feeding and does not confer epidemiologic importance. After a critical review of the evidence for and against tick transmission, we conclude that transmission of any *Bartonella* spp. by ticks, to animals or humans, has not been established. We are unaware of any well-documented case of *B. henselae* transmission by *I. scapularis* ticks.

Infections with *Bartonella* spp. appear to be widespread in many animal species besides cats (*1*). Some evidence has been advanced in support of the possibility of tick transmission. Such findings have resulted in diagnostic testing and empiric therapies directed at *B. henselae* infection that are of dubious value with respect to illnesses thought to be caused by deer tick exposure. We critically examined the reported findings regarding tick transmission of *Bartonella* spp.

Bartonella spp. are common bacterial hemoparasites of mammals; for as long as 100 years, 2 species have been known to cause infections of public health significance. Trench fever, caused by *B. quintana* (formerly *Rochalimaea quintana*) and transmitted by body lice, affected hundreds of thousands of soldiers or displaced persons during World War I and to this day affects homeless persons. Oroya fever (and its chronic manifestation verruga peruana), caused by infection with *B. bacilliformis* and transmitted by phlebotomine sandflies, is a potentially severe febrile disease. Although it is geographically restricted to the high altitudes of the Andes and affects only a relatively small number of persons, the high case-fatality rate brought attention to this apparent anthroponosis as early as the late 1800s.

B. henselae causes cat-scratch disease, the most common *Bartonella* spp. infection in the United States (*2*). The hallmark of cat-scratch disease is enlargement and tenderness of lymph nodes draining the site of inoculation of the microorganism (*3*). In addition, a skin or mucous membrane lesion may be observed at the site of inoculation for 25% to >90% of patients (*3*,*4*). Extranodal clinical manifestations (e.g., encephalopathy, neuroretinitis, arthritis, and lytic bone lesions) occur in ≈10% of patients (*3*–*6*). Cats are the main reservoir of *B. henselae*. In a study from San Francisco, 25 (41%) of 61 pet, pound, or stray cats (*Felis domesticus*) were found to have *B. henselae* bacteremia (*7*). Bites or scratches from infected cats are associated with development of cat-scratch disease. The gut of cat fleas is commonly infected, and exposure to feces of infected fleas is the presumed route of transmission to uninfected cats and a possible route of transmission to humans.

Parasitologists focusing on blood parasites have long noted the ubiquity of *Bartonella* spp. within mammals, particularly rodents, and by the late 1960s nearly 2 dozen species had been described within the genus *Grahamella* (*8*). The genera *Rochalimaea* and *Grahamella* were subsumed into the genus *Bartonella* (*9*), and many of the validly published *Grahamella* spp. have been excluded from the list of approved bacterial taxa (*10*). These actions tended to foster ignorance of the history of the diversity of *Bartonella* spp.

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DOI: 10.3201/eid1603.090443

and to promote a fallacy in pathogen discovery (*11*); namely, if a DNA sequence is not present in GenBank, surely it must represent something novel, the extensive classical literature on a likely identical organism known only by morphology notwithstanding. The significance of such a fallacy is that a large body of literature that may provide critical details on the biology of a "novel" agent is completely overlooked or dismissed.

Vector Relationships

Seminal studies by Richard Pearson Strong and the members of the American Red Cross trench fever commission (*12*) conclusively demonstrated biological as opposed to mechanical transmission of the trench fever agent by body lice. Feeding experiments on human volunteers established that lice may transmit by bite or by fecal contamination of abraded skin; that an infected louse remains infectious for at least 2 weeks; that the agent is not inherited by the progeny of infected lice; and that transmission may be extremely efficient, causing trench fever in 75% of volunteers after 1 exposure to a feeding box containing ≈50 lice that had previously fed on patients with trench fever.

Although initially Oroya fever was epidemiologically associated with ticks (*13*), it rapidly became evident that phlebotomine sandflies (particularly *Lutzomyia verrucarum*) were the vectors. Sandflies were the only blood-feeding arthropods that were peridomestic in their habits and occurred in the "bartonella zone," >2,000 m elevation. Experimentally, sandflies acquired infection from blood-smear positive patients and transmitted infection by bite to those without evidence of *Bartonella* spp. infection (*14*).

Grahamellae (now bartonellae) of rodents have long been known to be transmitted by fleas (*15–17*). Such studies have noted the difficulty with which experimental infections may be established by means other than inoculation of flea homogenates, the persistence within the rectal sac of the flea, and the likely mode of perpetuation of the bacteria by larval fleas ingesting dried infected blood. In addition, grahamellae-infected rodents were noted to exist in the absence of ticks, demonstrating that ticks were not required to perpetuate these particular bacteria.

Ticks as Vectors

Ticks are notorious vectors of a variety of agents that cause zoonotic infections (*11*), including viruses, bacteria, and protozoans. Like all animals, ticks have a diverse microflora. Recent analyses, using cloning and sequencing broad-range 16S rDNA amplification products, have documented a large bacterial flora within northeastern populations of *Ixodes scapularis* ticks that bite humans as nymphs, hereafter referred to as deer ticks (*18*,*19*). Amebas, mycoplasma, fungi, and helminths have been detected in these ticks by microscopy or other standard methods. However, the presence of a microbial agent within a tick does not imply that the tick might transmit it during the course of blood feeding or that it is pathogenic.

During early investigations of the causes of Oroya fever, Noguchi (*20*) demonstrated that *B. bacilliformis* could be experimentally transmitted between monkeys by the bites of *Dermacentor andersoni* ticks. However, the ticks that had been fed for a few days on infected monkeys were removed and allowed to reattach and complete their blood meal on uninfected animals, which became infected. Noguchi concluded that mechanical transmission had been demonstrated (perhaps by contamination of mouthparts or by regurgitation of the infectious partial blood meal), but persistence of viable bacteria or transstadial passage had not, and thus ticks were not biologic vectors.

Based on the volume of studies, the most compelling argument in favor of a tick vector for *Bartonella* spp. is that these microorganisms are sometimes detected in field-collected ticks (Table 1) (*15*). Although at least 20 studies have provided evidence for the presence of *Bartonella* spp. in primarily *Ixodes* spp. ticks collected at various locations in the United States and Europe, only 1 study has confirmed the presence of *Bartonella* spp. by culture (*15*,*21*,*22*). Caution is warranted when interpreting such data, however, because acquisition of *Bartonella* spp. from animal sources through a blood meal would be anticipated given the ubiquity of the microorganism in domestic animals and wildlife. In New England, as many as 60% of white-footed mice are blood-smear positive for *Grahamella* spp. (now *Bartonella*), regardless of collection site, including those trapped within the house of 1 of the authors where a tick life cycle was not present (S.R. Telford III, unpub. data); prevalence would probably reach unity if more sensitive modes of detection were used. The mere presence of *Bartonella* spp. or their DNA in ticks does not prove vector competence or

Table 1. Reasons that *Bartonella* species might be transmitted by ticks

- Certain other arthropods can transmit *Bartonella* species.
- Seropositivity to *B. vinsonii* subsp. *berkhoffii* in dogs correlates with tick exposure and with seropositivity to other tick-borne pathogens. Seropositivity to *B. henselae* in feral cats in the United Kingdom correlated with seropositivity to *Borrelia burgdorferi*.
- *Bartonella* spp. DNA is present in ticks.
- Cases of *B. henselae* infection with preceding tick bite have been reported.
- Transstadial transmission of *B. henselae* in *Ixodes ricinus* ticks and transmission by *I. ricinus* ticks during a blood meal using an artificial feeding system have been shown.
- Case control study of cat-scratch disease found a significant association with having had a tick on the body, but this association lost statistical significance on a bivariate analysis controlling for kitten exposure.
- *Bartonella* spp. are commonly present in *Peromyscus leucopus* mice, a major host for deer ticks and a main reservoir of *B. burgdorferi*.

confer epidemiologic significance (*15*), but it should serve as the impetus to rigorously perform the studies necessary to establish vector competence of ticks. At the least, viability should be established for bartonellae detected within ticks by means of in vitro cultivation.

To date, no report has documented transmission of *B. henselae* or any other *Bartonella* spp. to an animal after a tick bite (Table 2). The strongest evidence that ticks might be competent vectors for bartonellae was reported in a recent study in which *I. ricinus* ticks were infected with *B. henselae* in spiked (artificially infected) ovine blood by using an artificial feeding system (*23*). The ticks maintained infection throughout the molt, thereby establishing transstadial transmission. The experimentally infected ticks were also able to transmit *B. henselae* during a subsequent blood meal, again through the artificial feeding system; the dissected salivary glands from such ticks, when introduced into a cat, produced typical *B. henselae* infection, proving viability. Serious questions exist, however, as to whether these experiments are relevant to establishing vector competence. The ticks were fed continuously on blood meals with 10^9 CFU/mL, representing a bacteremia that would rarely be seen in natural infections of cats. Given that *Ixodes* spp. nymphs ingest a total of ≈15 μL blood (*24*), each nymph may have ingested 10^6–10^7 bacteria, a large dose. In addition, the Houston-1 strain of *B. henselae* used in this study may not represent strains found in nature. It is highly adapted to the laboratory and readily grows in vitro, whereas primary isolates are extremely fastidious and grow slowly.

A more straightforward experiment to establish vector competence would be to feed an uninfected *Ixodes* sp. tick on a *B. henselae*–infected cat and then, after the tick has molted, determine whether *B. henselae* can be transmitted by tick bite to an uninfected cat. However, even if such an experiment were to prove vector competence, additional data would be needed to conclude that *Ixodes* spp. ticks are epidemiologically relevant as *B. henselae* vectors.

Do epidemiologic data that support tick transmission of *Bartonella* spp. in animals exist? One study correlated canine seropositivity to *B. vinsonii* subsp. *berkhoffii* with tick exposure and with seropositivity to other tick-borne pathogens (*25*). However, the dogs in that study were also heavily exposed to fleas, and according to findings with cats, flea transmission is as likely a possibility as tick transmission in dogs, if not more so (*15*,*25*,*26*). A study in the United Kingdom reported an association between seropositivity to *B. henselae* and to *Borrelia burgdorferi* in feral cats (*27*). The method used to detect antibodies to *B. burgdorferi* was not precisely described. However, the fact that the rate of seropositivity to *B. henselae* was nearly the same for domestic and feral cats, despite domestic cats having much less tick exposure than feral cats, raises questions about the epidemiologic relevance of tick transmission. In another study, a "novel" *Bartonella* subspecies was detected more often in white-footed mice concurrently infected with the tick-borne pathogens *B. burgdorferi* or *Babesia microti* (*1*), but this analysis failed to compare the likelihood that the *Bartonella* spp. might also commonly co-occur with rodent trypanosomes, which are maintained by fleas. Epidemiologic arguments must carefully control for confounding, and none to date argues convincingly for tick transmission of *Bartonella* spp.

Table 2. Reasons that transmission of *Bartonella henselae* by deer ticks is unlikely or unproven

- Typical cat-scratch disease after a recognized deer tick bite has not been observed.
- Cat-scratch disease has a different seasonal pattern from that of Lyme disease.
- Appropriate seroepidemiologic studies have not been done.
- Vector competence of ticks for *B. henselae* in an animal system has not been proven.
- No convincing evidence of *B. henselae* in deer ticks has been reported.
- The *Bartonella* species present in *Peromyscus leucopus* mice is not *B. henselae*.
- The US cases with convincing evidence of *B. henselae* infection after a tick bite occurred in areas where Lyme disease is not endemic.

Studies of Humans

Certain authors have interpreted their studies as providing epidemiologic support for tick transmission of *Bartonella* spp. These data are, however, largely anecdotal and inconclusive (*28*,*29*). Culture-confirmed *B. henselae* infection was reported in 3 US patients who had been bitten by a tick within a few weeks of onset of illness (*28*,*30*); 2 of these patients had been in contact with a cat and may have been infected by this animal or its fleas. The tick species causing the bites was not identified for any of the patients but was unlikely to have been deer ticks because of the locations (Arkansas, Oklahoma, and probably North Carolina) (*30*), in which deer tick bites would be rare. *Bartonella* spp. have rarely (2 of ≈500 ticks) been detected in *Amblyomma americanum* ticks, the most common tick species to parasitize humans in these 3 states (*22*), but the finding was based on 1 PCR and not confirmed with a second target or any other assay.

A more recent study described 3 patients from Europe for whom a scalp eschar and neck lymphadenopathy were attributed to tick transmission of *B. henselae* (*31*). Molecular detection of the microorganism by PCR of a biopsy specimen from the eschar, in conjunction with a high serum antibody titer by immunofluorescence assay, document *B. henselae* infection for 2 of the patients; a tick bite at the lesion site was presumed but not proven for either patient. Both had been in contact with cats that may well have transmitted this infection because the clinical features were

indistinguishable from those of cat-scratch disease. The third patient, who had no cat exposure, had a documented bite from a *Dermacentor marginatus* tick that had PCR evidence of *B. henselae* infection. Whether the patient actually had *B. henselae* infection is questionable because PCR testing of tissue from the eschar was negative and antibodies to *B. henselae* could not be detected by immunofluorescence assay. The sole stated basis for the diagnosis was a positive Western blot result, but neither the interpretive criteria used nor the specificity of this testing were provided. When associated with a documented tick bite, the most common cause for a scalp eschar and neck lymphadenopathy is *Rickettsia slovaca*, but other rickettsia and even *Francisella tularensis* are possible causes, and in at least 25% of cases no pathogen can be identified (*31*).

Univariate analysis in a case–control study of cat-scratch disease in Connecticut found a significant association between having found a tick on the body and cat-scratch disease (*32*). This association, however, did not remain significant on multivariate (bivariate) analysis after controlling for exposure to kittens.

A 2001 report from New Jersey described 3 patients believed to have nervous system co-infection with *B. henselae* and *B. burgdorferi* (*33*). The authors suggested that bartonellae were transmitted by infected deer ticks because of the co-infection with *B. burgdorferi* and because the investigators detected *B. henselae* in a deer tick found in the household of 1 of these co-infected patients and in several deer ticks found on the pet cat of a fourth patient believed to have only *B. henselae* infection. PCR detection of DNA of both *B. burgdorferi* and *B. henselae* in the cerebrospinal fluid of these patients was the primary basis for the diagnosis of co-infection. An accompanying editorial, however, raised concerns about the validity of the diagnosis of both neuroborreliosis and neurobartonellosis in these patients (*34*). The clinical features were atypical for either infection, and the laboratory test results in support of these infections showed inconsistencies. In addition, 2 of the 3 authors had a potential conflict of interest; they were associated with a commercial laboratory that stood to gain financially from laboratory testing for *B. henselae*. The PCRs used by these investigators and others need careful scrutiny. In a later publication (*35*), the authors of the original NJ report conceded that the primers that they had used to amplify *B. henselae* DNA were insufficiently specific to warrant the conclusion that *B. henselae* was detected. BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) analysis of their primer P12B demonstrates identity with mouse mitochondrial DNA; also, what might be amplified if the PCR reaction were not stringent enough (e.g., lower annealing temperature) is not clear. In addition, their primer P24E contains a large proportion of α-proteobacterial 3′ terminus 16S rDNA consensus sequence. Because the specificity of PCR testing depends on target selection and reaction conditions, molecular detection using current primer sets may identify yet-undescribed genera of environmental bacteria distinct from *Bartonella* spp. Future examination of field-collected ticks for *Bartonella* spp. DNA should use a minimum of 2 independent PCR targets, preferably those that include larger portions of phylogenetically informative genes; to demonstrate viability, *Bartonella* spp. cultures should be attempted from all DNA-positive ticks. The deer ticks were unlikely to have been actually infected with *B. henselae* unless one postulates that feral cats serve as common hosts to larval or nymphal deer ticks. Indeed, the relatively high prevalence of reported *Bartonella* spp. infection (*35*) suggests that these ticks feed on cats as frequently as they do on mice. Although cats certainly serve as hosts for deer ticks of all stages, their contribution to feeding these vectors relative to all other animals remains to be defined and is likely to be minimal compared with rodents or birds. Given how frequently deer ticks feed on mice, *B. vinsonii arupensis* (previously known as *Grahamella peromysci*), which was isolated from a febrile, encephalopathic patient as well as from a patient who died from endocarditis, should more commonly infect persons in Lyme disease–endemic sites. This agent, however, has not been detected in deer ticks in any survey to date. Nevertheless, that *B. henselae* infection is a potential deer tick-transmitted co-infection in patients with possible Lyme disease is still widely accepted by the "chronic Lyme disease" counterculture (i.e., those physicians, patients, and activists who believe that patients with unexplained subjective symptoms have chronic *B. burgdorferi* infection even in the absence of exposure to a disease-endemic area or credible laboratory evidence of infection) (*36*).

Anecdotal accounts of *B. henselae* co-infection with *B. burgdorferi* in patients have been reported from Poland (*37*), Russia (*29*), and North Carolina (*38*). The report from North Carolina relied solely on immunoglobulin (Ig) M seroreactivity to *B. burgdorferi* to support a diagnosis of neuroborreliosis (*38*). The relatively poor specificity of IgM serologic testing (*39*) and the fact that the case was from outside Lyme disease–endemic regions of the United States raise concerns about the validity of the diagnosis of *B. burgdorferi* infection in this patient.

A straightforward approach to address whether *B. henselae* is transmitted by deer ticks would be seroepidemiologic studies to compare the prevalence of *B. henselae* antibodies in patients with Lyme disease with those in appropriate control groups, but such studies have not been performed. A study in Slovenia found that only 1 of the 86 children in whom febrile illness developed after a tick bite had Lyme disease in conjunction with seroconversion for IgG antibodies to both *B. henselae* and *B. quintana* (*40*).

In the United States alone, >20,000 cases of Lyme disease and about the same number of cases of cat-scratch

disease occur annually (*2*). Thus, co-infections may occur occasionally by chance alone, without cotransmission by a tick vector. If the bite of a deer tick is a common route for *B. henselae* transmission, the absence of reports of the typical lymph node findings of cat-scratch disease proximal to the bite site of this tick species seems puzzling. The seasonality of cat-scratch disease, in which most cases in temperate regions occur in autumn and early winter (when peak breeding of cat fleas and birth of kittens occur), provides further evidence against a major role for ticks in transmission of *B. henselae* (*32*).

Conclusion

Tick transmission of any *Bartonella* spp. to either animals or humans has not been established. *B. henselae* in particular is unlikely to be transmitted by deer ticks, and, to our knowledge, no well-documented case of transmission by this tick species in humans or animals has been reported.

Acknowledgments

We thank Lisa Giarratano and Lenise Banwarie for their assistance.

S.R.T. is supported by National Institutes of Health RO1 AI 064218.

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Potential for Tick-borne Bartonelloses

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As worldwide vectors of human infectious diseases, ticks are considered to be second only to mosquitoes. Each tick species has preferred environmental conditions and biotopes that determine its geographic distribution, the pathogens it vectors, and the areas that pose risk for tick-borne diseases. Researchers have identified an increasing number of bacterial pathogens that are transmitted by ticks, including *Anaplasma*, *Borrelia, Ehrlichia*, and *Rickettsia* spp. Recent reports involving humans and canines suggest that ticks should be considered as potential vectors of *Bartonella* spp. To strengthen this suggestion, numerous molecular surveys to detect *Bartonella* DNA in ticks have been conducted. However, there is little evidence that *Bartonella* spp. can replicate within ticks and no definitive evidence of transmission by a tick to a vertebrate host.

Bartonella spp. are gram-negative bacilli or coccobacilli that belong to the α-2 subgroup of *Proteobacteria.* According to 16S rDNA gene comparisons, they are closely related to the genera *Brucella* and *Agrobacterium* (*1*). A remarkable feature of the genus *Bartonella* is the ability of a single species to cause either acute or chronic infection that can cause either vascular proliferative lesions or suppurative and granulomatous inflammation. The pathologic response to infection with *Bartonella* spp. varies substantially with the status of the host's immune system; vasoproliferative lesions are most frequently reported for immunocompromised patients. To date, 13 *Bartonella* species and subspecies have been associated with an increasing spectrum of clinical syndromes in humans, including cat-scratch disease and chronic bacteremia (*B. henselae*), bacillary angiomatosis (*B. henselae*, *B. quintana*), peliosis hepatitis (*B. henselae*), bacteremia and/or endocarditis (*B. henselae*, *B. quintana, B. elizabethae*, *B. vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *berkhoffii*, *B. koehlerae*, and *B. alsatica*), Carrión disease (*B. bacilliformis*), trench fever (*B. quintana*), retinitis and uveitis (*B. henselae*, *B. grahamii*), myocarditis (*B. vinsonii* subsp. *berkhoffii*, *B. washoensis*), splenomegaly (*B. bacilliformis, B. henselae, B. rochalimae*), and fever and fatigue (*B. henselae, B. vinsonii* subsp. *berkhoffii*, *B. tamiae*) (*1*–*3*).

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DOI: 10.3201/eid1603.091685

Ticks

Ticks were first identified as potential vectors of *Babesia bigemina,* the agent of Texas cattle fever, in 1893 (*4*). There are 2 major tick families (≈865 tick species worldwide): the Ixodidae, or hard ticks, characterized by a sclerotized dorsal plate, and the Argasidae, or soft ticks, characterized by their flexible cuticle. A third family, the Nuttalliellidae, is represented by a single species that is confined to southern Africa. The genus *Ixodes*, family Ixodidae, contains >200 species, of which 14 make up the *I. ricinus* complex (*4*). Among these 14 species, *I. scapularis*, *I. pacificus*, *I. ricinus*, and *I. persulcatus* ticks are involved in the transmission of the *Borrelia burgdorferi* complex, which is a prevalent cause of Lyme disease in persons in the Northern Hemisphere.

Ticks in various regions of the world are vectors for bacterial, viral, and protozoal pathogens (*5*). Ticks may act not only as vectors but also as reservoirs of tick-transmitted bacteria that are transmitted transstadially and transovarially in a tick species (e.g., certain *Rickettsia* spp. and *Borrelia* spp.) (*5*). When feeding on an infected small-mammal host, larvae and nymphs can ingest ≥1 pathogens while

obtaining a blood meal. Some organisms are then passaged to the next stage in the tick life cycle and can be transmissible during the subsequent blood meal (*5*). For each tick species, the optimal environmental conditions determine the geographic distribution; the spectrum of tick-borne pathogens; and as a result, the geographic areas of risk for tick-borne diseases, particularly when ticks are both vectors and reservoirs of specific pathogens.

Hard ticks are the primary vectors of a variety of bacterial pathogens, including *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp., *Coxiella burnetii*, and *Rickettsia* spp (*5*–*7*). *Anaplasma phagocytophilum* is transmitted by *I. persulcatus*–complex ticks, including *I. scapularis*, *I. pacificus,* and *I. ricinus*, whereas *Ehrlichia chaffeensis* and *Ehrlichia ewingii* are transmitted by *Amblyomma americanum* ticks (*5*,*6*). Although some pathogens are carried by a single or limited number of tick species, other organisms such as *Coxiella burnetii* have been identified in >40 tick species (*7*). Lyme disease, caused by *B. burgdorferi*, is transmitted by *I. scapularis* and *I. pacificus* ticks within the United States, by *I. ricinus* ticks in Europe, and by other *Ixodes* spp. ticks in the Northern Hemisphere (*5*,*8*). Although specific *Bartonella* spp. are transmitted by blood-sucking arthropods, including fleas, lice, or sandflies, the only evidence to support the possibility of tick-borne transmission is indirect.

We present an overview of the various *Bartonella* spp. that have been detected in ticks and discuss human cases of *Bartonella* infection that are suggestive of tick transmission. Because of the rapidly expanding number of reservoir host–adapted *Bartonella* spp. that have been discovered in recent years, efforts to clarify modes of transmission are relevant to public health in terms of interrupting the transmission process. As evolving evidence supports the ability of this genus to induce chronic intravascular infections in humans, improved understanding of vector competence could facilitate efforts to block pathogen transmission, which would help improve human health (*9*).

Host Associations and Specificity

Bartonella spp. have a natural cycle of chronic intravascular infection in a reservoir host and a sustained pattern of bacterial transmission by a defined and evolutionarily well-adapted vector from the reservoir hosts to new susceptible hosts. Current information leads to the presumption of a long-standing and highly adapted species-specific association between a given *Bartonella* sp. and the preferred animal host and vector (*10*). Inadvertent infection of persons with at least 13 *Bartonella* spp. has resulted in a wide spectrum of disease manifestations. After primary infection of the natural mammalian host, a chronic, relapsing, nonclinical bacteremia occurs. At times, in wild and stray animal populations, including cats, cows, and various rodent species, the prevalence of infection within the population can approach 100% (*1*). Although the geographic distribution of a specific *Bartonella* sp. may reflect the geographic distribution of its hosts or vectors, knowledge related to vector transmission of *Bartonella* organisms remains inadequate.

Bartonella spp. DNA in Ticks

As an initial effort to define tick species that might serve as competent vectors for transmission of *Bartonella* spp., molecular epidemiology surveys to identify *Bartonella* spp. DNA in ticks have been conducted (*2*). *Bartonella* spp. have mostly been identified by PCR using primers targeting either specific *Bartonella* genes like the citrate synthase gene (*glt*A) gene, the riboflavin synthase gene, the heat shock protein gene (*gro*EL), the 16S–23S intergenic spacer, the heme binding protein gene, and the cell division protein gene or the 16S rDNA gene (Table 1). Summarized results indicate that the proportion of ticks harboring *Bartonella* DNA can vary from low prevalences of 0.43% among questing *A. americanum* ticks examined in the southeastern United States (*3*) and 1.2% of *I. ricinus* ticks collected in the Czech Republic (*24*) to a prevalence as high as 60% in *I. ricinus* ticks from roe deer in the Netherlands (*20*) (Table 1). *Bartonella* spp. from various locations tend to differ. For example, *Bartonella* DNA related to *B. doshiae, B. rattimassiliensis*, and *B. tribocorum* has been identified in ticks only in Asia, *B. bacilliformis*–like DNA and *B. capreoli* in ticks only in Europe, and *B. washoensis, B. tamiae*–like DNA, and *B. vinsonii* subsp. *berkhoffii* in ticks only in the United States (Figure).

Evidence for Co-infections in Ticks

In recent years, emphasis on the potential transmission of multiple pathogens by an individual tick after attachment to an animal or person has grown. While studying different tick populations throughout the world, several researchers have identified *Bartonella* DNA in conjunction with known tick-transmitted organisms. Adelson et al. tested for the prevalence of *B. burgdorferi*, *Babesia microti*, *A. phagocytophilum*, and *Bartonella* spp. in 107 *I. scapularis* ticks collected in New Jersey (*27*). A large percentage of ticks (45.8%) contained DNA from at least 1 of these organisms, and 34.5% of ticks screened harbored *Bartonella* spp. DNA. Of the ticks positive for *Bartonella* by PCR, 9 (8.4%) contained *B. burgdorferi* DNA, 1 (0.9%) contained *B. microti* DNA, 1 (0.9%) contained *A. phagocytophilum* DNA, 1 (0.9%) contained both *B. burgdorferi* and *A. phagocytophilum* DNA, and 1 (0.9%) contained *B. microti* and *A. phagocytophilum* DNA (*27*). Although the primers in this study were originally selected for the species-specific amplification of *B. henselae*, this region of the *Bartonella* 16S rDNA gene is highly conserved among many *Bartonella*

spp. In a study performed in France, Halos et al. screened 92 questing *I. ricinus* ticks and determined that 9.8% contained *Bartonella* DNA by using *glt*A-specific primers (*22*). *Bartonella schoenbuchensis*–like DNA (96% homology) was detected in 1 of the adult ticks tested. The authors also reported that 1% of the ticks contained *Bartonella* spp. and *B. burgdorferi* DNA, 4% contained *Bartonella* and *Babesia* spp. DNA, and 1% contained *Bartonella* spp., *B. burgdorferi*, and *Babesia* spp. DNA (*22*). Of 168 questing adult *I. pacificus* ticks from Santa Cruz County, California, screened for *Bartonella* DNA, 11 (6.55%) contained *B. henselae* genotype I DNA (*31*). Of the *Bartonella*–positive ticks, 1.19% also harbored *B. burgdorferi* DNA and 2.98% harbored *A. phagocytophilum* DNA (*31*). Loftis et al. tested *Carios kelleyi* ticks, argasid tick species found on bats, from residential and community buildings in Iowa, for *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, and *Rickettsia* spp. One tick was found to contain *Bartonella* and *Rickettsia* DNA, and the DNA sequence was most closely related to *B. henselae* (*11*). Recently, Sun et al. examined *Haemaphysalis longicornis* and *I. sinensis* from the People's Republic of China for *Borrelia*, *Bartonella*, *Anaplasma*, and

Table 1. Ticks in which *Bartonella* spp. DNA has been found*

Tick genus and species	Prevalence of *Bartonella* spp. DNA in ticks, %/no.	Identified *Bartonella* spp.	Target gene	Reference
Amblyomma americanum	0.43/466 individuals	*B. tamiae*–like	IGS	(*3*)
Carios kelleyi	3.2/31 individuals	Resembling *B. henselae*	IGS	(*11*)
Dermacentor occidentalis	8.3/12 pools	*Bartonella* spp.	*glt*A	(*12*)
D. reticulatus	21.4/84 individuals	*B. henselae* (99% homology) and *B. quintana* (90% homology)	*gro*EL	(*13*)
D. variabilis	14.3/ 7 pools	*Bartonella* spp.	*glt*A	(*12*)
Haemaphysalis flava	2.7/74 pools	*Bartonella* spp.	16S rRNA	(*14*)
H. longicornis	4.4/1,173 pools	*Bartonella* spp.; 1 pool harbored *B. rattimassiliensis* (99.2%), 1 pool harbored *B. tribocorum* (98.3%)	16S rRNA	(*14*)
H. longicornis	36/150 groups (60 individual fed adults, 30 pools of 2 unfed adults, and 60 pools of 5 nymphs)	*Bartonella* spp.	*glt*A	(*15*)
Ixodes nipponensis	5.0/20 pools	*Bartonella* spp.	16S rRNA	(*14*)
I. pacificus	19.2 of 151 individuals	*B. henselae, B. quintana, B. washoensis, B. vinsonii subsp. berkhoffii*, and a *Bartonella* cattle strain	*glt*A	(*16*)
I. pacificus	11.6/224 pools	*Bartonella* spp.	*glt*A	(*12*)
I. persulcatus	37.6/125 individuals	*B. henselae* (99% homology) and *B. quintana* (90% homology)	*gro*EL	(*13*)
I. persulcatus	44/50 individuals in 2002 and 38/50 individuals in 2003	*B. henselae*	*gro*EL	(*17*)
I. persulcatus	33.3/3 pools	*Bartonella* spp.	16S rRNA	(*14*)
I. ricinus	1.48/271 individuals	*B. henselae*	*gro*EL, *pap*31, *fts*Z	(*18*)
I. ricinus	4.9/102 individuals	*B. henselae*	*glt*A	(*19*)
I. ricinus	60/121 individuals	*Bartonella* spp	16S rRNA	(*20*)
I. ricinus	A pool/12 ticks	*Bartonella* spp	16S rDNA	(*21*)
I. ricinus	9.8/92 individuals	*Bartonella* spp.; 1 adult harbored *B. schoenbuchensis* (96% homology)	*glt*A	(*22*)
I. ricinus	7.7/103 individuals	*B. capreoli*	ITS	(23)
I. ricinus	1.2/327 individuals	*Bartonella* spp	16S rRNA	(*24*)
I. ricinus		Resembling *B. bacilliformis*†		(*25*)†
I. scapularis	2.0/203 individuals	*B. schoenbuchensis*	*glt*A	(*26*)
I. scapularis	34.5/107 individuals	Unidentified *Bartonella* spp.	16S rRNA	(*27*)
I. scapularis		*B. henselae*	16S rRNA	(*28*)
I. sinensis	16.3/86 individuals	*Bartonella* spp.	*glt*A	(*15*)
I. spp.	42.3/26 pools	*Bartonella* spp.	16S rRNA	(*17*)
I. turdus	11.1/9 pools	*Bartonella* spp.; 1 pool harbored *B. doshiae* (99.2% homology)	16S rRNA	(*14*)
Rhipicephalus sanguineus	3.2/62 individuals	*B. henselae*	*rib*C	(*29*)
Unidentified tick species		*Bartonella* sp.	IGS	(*30*)

*IGS, intergenic spacer; *glt*A , citrate synthase gene; *gro*EL, heat-shock protein gene; *pap*31, heme-binding protein gene; *fts*Z , cell-division protein gene; *rib*C, riboflavin synthase gene.
†*Bartonella* spp. ascertained by isolation.

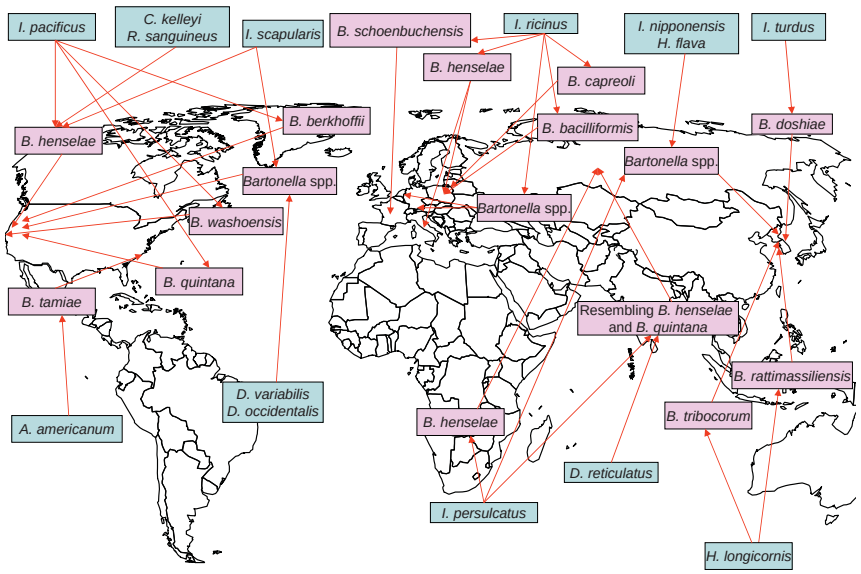

Figure. Worldwide locations of ticks (blue boxes) identified with *Bartonella* spp. (pink boxes). *I.*, *ixodes; C.*, *Carios*; *R.*, *Rhipicephalus*; *B.*, *Bartonella*; *H.*, *Haemaphysalis*; *A.*, *Amblyomma*; *D.*, *Dermacentor.*

Erhlichia spp. (*15*). Of adult and nymphal *H. longicornis* ticks collected in the cities of Benxi and Liaoyang, 36% of 150 groups (60 individual host-associated adults, 30 pools of 2 questing adults, and 60 pools of 5 nymphs) harbored detectable *Bartonella* DNA. Furthermore, 16.3% of 86 individual *I. sinensis* ticks (all host-associated adults) from the cities of Tiantai, Jindong, and Jiangshan contained *Bartonella* DNA. One tick harbored all 4 bacteria (*Borrelia*, *Bartonella*, *Anaplasma*, and *Ehrlichia* spp. DNA), and a second tick pool was positive by PCR for *Borrelia*, *Bartonella*, and *Ehrlichia* spp (*15*).

Evidence of Potential Tick *Bartonella* spp. Transmission to Humans

In 1992, *B. henselae* infection developed in 2 previously healthy, immunocompetent men within weeks of a tick bite (*32*) (Table 2). Both patients reported signs and symptoms generally associated with *B. henselae* infection: fever, muscle and joint pain, headache, and photophobia. The first patient did not recall being bitten or scratched by a cat, the general mode of *B. henselae* transmission to humans. *B. henselae* organisms were cultured from the blood of both patients and confirmed by PCR. To our knowledge, this was the first case report to suggest that ticks may be responsible for transmission of *Bartonella* spp. in humans. More recently, *B. henselae* was isolated from a boy who had severe intractable migraine headaches 10 days after an attached tick was removed from his leg, although on the basis of seroconversion, infection with *B. vinsonii* subsp. *berkhoffii* was suspected (*9*). Breitschwerdt et al. concluded that the boy was either co-infected or chronically infected with *B. henselae*, the organism isolated, and subsequently infected with *B. vinsonii* subsp. *berkhoffii*, as reflected by the documentation of seroconversion.

In a clinical study, Zangwill et al. were interested in identifying risk factors associated with development of cat-scratch disease (*33*). The epidemiologic survey, performed in Connecticut, contained 56 cat-scratch disease patients and their controls (persons who owned or had been in contact with cats). They used a modified random-digit dialing technique to recruit controls, and they identified 60 patients with cat-scratch disease. However, of the 60 patients whose illnesses met the case definition, 4 were not successfully matched with controls for age and cat ownership; therefore, 56 patients and their controls were enrolled in the case–control study. The controls did not differ significantly from the patients by race, sex, family size, level of maternal education, or socioeconomic status. Answers to questionnaires suggested that cat-scratch disease was more likely to occur in patients than in controls if the person owned a kitten, had contact with a kitten with fleas, or had been bitten or scratched by a kitten. Of the 56 patients, 21% were also more likely than controls to have been bitten by a tick, although bivariate analysis did not demonstrate a significant association between tick bite and cat-scratch disease development (*33*).

Other case reports have suggested potential human coinfections with *Bartonella* spp. and a known tick-transmitted organism. Eskow et al. described 4 cases in which patients from central New Jersey reported several neurologic symptoms, including headache, fatigue, insomnia, and depression, which may have resulted from Lyme disease (caused by *B. burgdorferi*) (*28*). However, other causes for their cognitive dysfunctions cannot be ruled out. Of these 4 patients, 2 had histories of Lyme disease, and 3 had *B. burgdorferi* DNA in the cerebrospinal fluid (CSF). One patient exhibited no laboratory evidence of Lyme disease, suggesting that these symptoms might have been caused

Table 2. Evidence of *Bartonella* spp. infection in persons after tick bite

Agent	Tick species	Tick bite	Animal contact	Clinical manifestation	Year	Reference
B. henselae	Unknown	Yes	No cat	Fever, myalgia, arthralgia, headaches, and light sensitivity	1992	(*32*)
B. henselae	Unknown	Yes	Cat	Fever, myalgia, arthralgia, headaches, and light sensitivity	1992	(*32*)
B. henselae	Unknown	Yes	Cats and kitten	Cat-scratch disease signs	1993	(*33*)
B. henselae, *Borrelia burgdorferi*	Possibly *Ixodes scapularis*	Yes	Not mentioned	Low-grade fever, headaches, fatigue, knee arthralgia, and insomnia	2001	(*28*)
B. henselae, B. burgdorferi	Possibly *I. scapularis*	Yes	Not mentioned	Fever, headache, dizziness, fatigue, and arthralgia	2001	(*28*)
B. henselae, B. burgdorferi	Unknown	Not mentioned	Not mentioned	Meningitis	2003	(*34*)
B. henselae or *B. quintana* seroreactive	Unknown	Yes	Not mentioned	Fever	2003	(*35*)
B. burgdorferi, *B. henselae*, *B. quintana*	Unknown	Yes	Not mentioned	Fever	2003	(35)
Bartonella spp. closely related to *B. henselae*, *B. quintana*	Unknown	Yes			2005	(*36*)
B. henselae and/or *B. vinsonii* subsp. *berkhoffii**	Unknown	Yes	Cats, dogs, potentially other animal species	Fatigue, insomnia, arthralgia, myalgia, headache, and/or tremors	2007	(*37*)
B. henselae, and/or *B. vinsonii* subsp. *berkhoffii*†	Unknown	Yes	Cats, dogs, other animal species	Seizures, ataxia, memory loss, tremors, fatigue, and/or headaches	2008	(*9*)

*Patients were also seroreactive to *B. henselae* and/or *B. vinsonii* subsp. *berkhoffii.*
†Patients were also seroreactive to *B. henselae*, *B. vinsonii* subsp. *berkhoffii,* and/or *B. quintana*.

by an agent other than *B. burgdorferi*. However, 2 patients reported illness within 1 week to 3 months after being bitten by a tick. Upon further investigation, all patients were seroreactive to *B. henselae*; immunofluorescence assay showed immunoglobulin (Ig) G titers of 64–256. According to the authors, *B. henselae* DNA was amplified from blood of 1 patient, from CSF of 1 patient, and from both blood and CSF of the other 2 patients (*B. burgdorferi* DNA also was detected in the CSF of these 2 patients). Ticks, identified as *I. scapularis*, found in 2 patients' homes potentially harbored both *B. henselae* and *B. burgdorferi* DNA. Whether *B. henselae* was specifically detected in this case series is unclear because sequencing of amplicons was not performed and because the PCR primer set targeted the *Bartonella* 16S rRNA, a highly conserved region. Without sequencing of amplicons or confirmation of results by targeting a more highly variable gene, ascertaining whether *B. henselae* was present in the ticks or in the patients would be difficult. However, the results derived from these cases are of interest because, to our knowledge, this was the first case series to propose simultaneous detection of both *B. burgdorferi* and *Bartonella* DNA in the CSF of patients with neurologic signs.

In another study, 2 of 17 patients from Poland with symptoms suggestive of neuroborreliosis seemed to be co-infected with *B. burgdorferi* and *B. henselae* (*34*). *B. burgdorferi*–specific antibodies were detected in a patient whose CSF also had detectable *B. henselae* DNA. The other patient was seroreactive to both *B. burgdorferi* and *B. henselae* antigens at titers of 32. The authors speculated that co-infection may be tick transmitted; however, contact with other arthropod species should be considered. Although the detection of *B. henselae* DNA in the CSF of these patients could be attributed to amplification of DNA from nonviable organisms or to laboratory error, the repeated documentation of *B. henselae* in blood and in CSF of a young woman with a previous diagnosis of classical cat-scratch disease support the potential that this bacterium can cause chronic intravascular and central nervous system infections in immunocompetent persons (*9*).

In a study performed in Slovenia, 86 febrile children were screened for serologic evidence of exposure to multiple tick-borne organisms within 6 weeks of a known tick bite (*35*). Acute- and convalescent-phase serum samples were collected from each child. Prior exposure was determined for 5 children who harbored *B. henselae* IgG and for 4 children who harbored *B. quintana* IgG. Seroconversion of IgG to both antigens was detected for only 1 child (*35*). Morozova et al. tested for *Bartonella* DNA in persons from the Novosibirsk region of Russia who had been bitten by ticks during the summers of 2003 and 2004 (*38*). *Bartonella* DNA closely related to *B. henselae* and *B. quintana* was detected in the blood of some patients by using *gro*EL-specific primers (*36*). A more recent study, performed by

Breitschwerdt et al., screened 42 immunocompetent patients, who had had prior animal and arthropod contact, for *Bartonella* spp. (*37*) The study included 12 women and 2 men who reported having had occupational animal contact for >10 years, including frequent animal bites, animal scratches, and arthropod exposure (e.g., fleas, ticks, biting flies, mosquitoes, lice, mites, chiggers). *B. henselae* or *B. vinsonii* subsp. *berkhoffii* were detected by PCR or were cultured from all patients (*37*). Case studies and surveys of this type suggest that ticks may serve as competent vectors of *Bartonella* spp., but this supposition cannot be confirmed until experimental studies demonstrating successful transmission have been performed.

Recently, Cotté et al. detailed the potential transmission of *B. henselae* by *I. ricinus* ticks (*38*). Using an artificial feeding platform made of rabbit skin, the authors successfully (based on PCR screening) infected ticks with *B. henselae* of molted ticks previously fed infected blood, suggesting that transstadial transmission may be possible. Subsequently, molted ticks were placed onto rabbit skins and fed noninfected blood, after which *B. henselae* was either cultured or detected by PCR analysis within 72 hours of when aliquots were taken from the previously noninfected blood. This finding indicates that during a blood meal, the organism could potentially be transferred from an infected tick to a noninfected individual. In addition, *B. henselae* bacteria were also present within molted ticks in sufficient numbers to cause bacteremia when tick salivary gland extracts were inoculated intravenously into domestic cats. Because ticks were not allowed to attach directly to the cats, this study supports, but does not prove, tick transmission of *B. henselae* by *I. ricinus*. Consistent with the transmission of *Bartonella* spp. by other arthropods such as fleas and lice, *B. henselae* does not seem to be transovarially transmitted in ticks because larvae hatched from *B. henselae*–positive (by PCR) egg clutches did not harbor detectable *Bartonella* DNA (*2*,*38*).

Conclusions

The number of zoonotic *Bartonella* spp. identified in the past 15 years has increased considerably. This review indicates that a diversity of *Bartonella* spp. DNA can be amplified from various tick species from numerous geographic locations, that tick attachment has preceded the onset of illness in a small number of patients from whom *B. henselae* DNA has been amplified, and that serologic and molecular evidence suggests cosegregation of *Bartonella* spp. with known tick-borne pathogens. Therefore, ticks might serve as potential *Bartonella* vectors. However, there is little evidence that *Bartonella* spp. can replicate within ticks and no definitive evidence of transmission by a tick to a vertebrate host. Only Kruszewska and Tylewska-Wiezbanowska reported successful isolation of *Bartonella* sp. from a tick (*25*); all other studies were based on amplification of *Bartonella* DNA from ticks by using PCR. As the medical relevance of the genus *Bartonella* continues to evolve, it is clearly necessary to determine whether ticks or other arthropods play a role in the transmission of *Bartonella* spp. among animals and humans. For this reason, experimental transmission studies, using infected ticks placed on live animals, are required to determine whether ticks are vector competent for the transmission of *Bartonella* spp.

Addendum

Since the submission of this manuscript, we found 3 cases of *B. henselae* infection transmitted by *Dermancentor* spp. ticks. These patients had scalp eschar and neck lymphadenopathy (*39*).

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Serologic Markers for Detecting Malaria in Areas of Low Endemicity, Somalia, 2008

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Areas in which malaria is not highly endemic are suitable for malaria elimination, but assessing transmission is difficult because of lack of sensitivity of commonly used methods. We evaluated serologic markers for detecting variation in malaria exposure in Somalia. *Plasmodium falciparum* or *P. vivax* was not detected by microscopy in cross-sectional surveys of samples from persons during the dry (0/1,178) and wet (0/1,128) seasons. Antibody responses against *P. falciparum* or *P. vivax* were detected in 17.9% (179/1,001) and 19.3% (202/1,044) of persons tested. Reactivity against *P. falciparum* was significantly different between 3 villages ($p<0.001$); clusters of seroreactivity were present. Distance to the nearest seasonal river was negatively associated with *P. falciparum* ($p = 0.028$) and *P. vivax* seroreactivity ($p = 0.016$). Serologic markers are a promising tool for detecting spatial variation in malaria exposure and evaluating malaria control efforts in areas where transmission has decreased to levels below the detection limit of microscopy.

Sub-Saharan Africa has the highest incidence of malaria caused by *Plasmodium falciparum*. Almost all areas where *P. falciparum* parasite prevalence is >50% in the general population are located in Africa (*1*). However, malaria is not uniformly distributed (*1*,*2*) and many parts of Africa are characterized by low transmission intensity of malaria (*1*). These areas are considered suitable for intensive malaria control and disease elimination (*3*,*4*).

Assessing malaria transmission intensity and evaluating interventions are complicated at low levels of malaria transmission. Assessing transmission intensity directly by determining the exposure to malaria-infected mosquitoes (entomologic inoculation rate [EIR]) is difficult when mosquito numbers are low, sometimes below the detection limits of commonly used trapping methods (*5*,*6*), and spatial and temporal variations in mosquito densities necessitate long-term intensive sampling (*5*,*7*,*8*). Determination of malaria parasite prevalence in the human population is a commonly used alternative (*9*), but it also becomes less reliable as an indicator of transmission intensity when endemicity is low (*3*,*9*,*10*). Therefore, an alternative method is needed to assess transmission intensity, evaluate interventions, and obtain information for control programs in areas of low endemicity.

Prevalence of antibodies against malaria parasites has been explored as a means of assessing malaria transmission intensity (*11*–*13*). Antibody seroconversion rates are less susceptible to seasonal fluctuations in malaria exposure (*11*,*12*), show a tight correlation with EIR (*12*,*13*), and show potential to detect recent changes in malaria transmission intensity (*14*). Serologic markers could be particularly useful in areas of low endemicity, where it may be easier to detect relatively long-lasting antibody responses than a low prevalence of malaria infections in the human population or infected mosquitoes. We used serologic markers of exposure to determine spatial variation in malaria transmission intensity in an area of low endemicity in Somalia (*15*).

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DOI: 10.3201/eid1603.090732

Methods

Study Area

This study was conducted in the Gebiley District in Somaliland in northwestern Somalia. The district has a predominantly arid landscape with a few seasonal rivers and patches of irrigated farmlands. It is an area of intense seasonal rainfall with an average annual precipitation of 59.9 mm (2004–2007) and 2 peaks in rainfall in April and August. Three moderately sized communities were randomly selected from census maps by using spatial random sampling techniques in Arcview version 3.2 (Environmental Systems Research Institute, Redlands, CA, USA) (*16*). These communities were the villages of Xuunshaley (9.72140°N, 43.42416°E), Badahabo (9.68497°N, 43.65616°E), and Ceel-Bardaale (9.81777°N, 43.47455°E). The research protocol was reviewed and approved by the Research Ethics Review Committee of the World Health Organization (RPC246-EMRO) and the Ethical Committee of the Ministry of Health and Labor, Republic of Somaliland.

Data Collection

Two cross-sectional surveys were conducted. The first survey was conducted in March 2008 to determine parasite carriage at the end of the dry season (*16*). The purpose of the survey and the procedures were first discussed with the clan elders; thereafter, each household was visited, and informed consent was sought from each head of household. Households that agreed to participate were geolocated by using a global positioning system (Garmin eTrex; Garmin International, Inc., Olathe, KS, USA), and information was collected on demographic characteristics, bed net use, and travel history of the participants. Distance to seasonal rivers or other water bodies and distance to the nearest livestock enclosure was determined by using the global positioning system.

Individual written consent was obtained from all literate adults; illiterate adults provided consent by a thumbprint in the presence of an independent literate adult witness. For children <18 years of age, consent was obtained from parents or guardians, and children 12–18 years of age who could not write also provided consent by a thumb print.

One fingerprick blood sample was obtained from each respondent for the preparation of a *P. falciparum* antigen–specific rapid diagnostic test (RDT) (Paracheck-Pf; Orchid Biomedical Systems, Goa, India) sample and thick and thin blood smears. One-hundred high-power microscopic fields were examined and an additional 100 fields were examined if the first 100 fields were negative. RDT results were used for treatment with sulfadoxine-pyrimethamine and 3 doses of artesunate according to national guidelines. A second cross-sectional survey was conducted at the end of the wet season (August–September 2008) by using procedures identical to those described above, except that part of the fingerprick blood sample was placed on filter paper (3 MM; Whatman, Maidstone, UK) as described by Corran et al. (*17*).

Entomologic Surveys

Presence of *Anopheles* spp. mosquitoes in the area was determined by larvae collections in all permanent water bodies (artificial rain water reservoirs, wells, boreholes, stagnant storage pits, and riverbeds) in the 3 villages at the end of the wet season. Locally produced 250-mL dippers with a white surface were used. Five to 10 dips were made in the large water bodies and the presence of *Anopheles* spp. larvae was visually assessed and recorded.

Elution of Serum

Filter paper samples were stored at 4°C with desiccant until processed. A 3.5-mm blood spot, equivalent to ≈3 μL of blood (*17*), was punched from the filter paper and placed in a labeled well of a low-binding 96-well titer plate. A total of 300 μL of reconstitution buffer (phosphate-buffered saline [PBS], 0.05%Tween, and 0.1% [wt/vol] sodium azide) was added, and plates were sealed and rocked gently at room temperature overnight and subsequently stored at 4°C. The reconstituted blood spot solution was equivalent to a 1:100 dilution of whole blood or a 1:200 dilution of serum.

ELISAs

All reconstituted filter paper spots were tested at a final serum dilution equivalent of 1:1,000 for human immunoglobulin G antibodies against *P. falciparum* merozoite surface protein 1_{19} (MSP-1_{19}) and 1:2,000 for antibodies against apical membrane antigen 1 (AMA-1) by using described ELISA methods (*12*,*17*). Briefly, recombinant MSP-1_{19} (Wellcome genotype) and AMA-1 (3D7) were coated overnight at 4°C at a concentration of 0.5 μg/mL. Plates were washed by using PBS, 0.05% Tween 20 (PBS/T) and blocked for 3 h with 1% (wt/vol) skim milk powder in PBS/T. Positive controls (a pool of hyperimmune serum) and negative controls (European malaria-negative volunteers) were added in duplicate to each plate. The plates were washed and horseradish peroxidase–conjugated rabbit anti-human immunoglobulin G (Dako, Roskilde, Denmark) (1:5,000 dilution in PBS/T) was added to all wells. Plates were developed for 20 min by using an *o*-phenylenediamine dihydrochloride substrate solution. Reactions were stopped with 2 mol/L H_2SO4. Plates were read immediately at 492 nm and optical density (OD) values recorded. For *P. vivax*, an identical protocol was used with MSP-1_{19} (0. 5 μg/mL) (*18*) and AMA-1 (0. 5 μg/mL). Serum in this protocol was used at 1:1,000 dilutions for both antigens.

Data Management and Statistical Analyses

Data were double-entered and imported into STATA version 10 (StataCorp LP, College Station, TX, USA). Duplicate OD results were averaged and normalized against the positive control sample on each plate. A cutoff value above which samples were considered antibody positive was defined by using a mixture model as described (*17*). Distribution of normalized OD values was fitted as the sum of 2 Gaussian distributions by using maximum-likelihood methods. The mean OD of the Gaussian distribution corresponding to the seronegative population plus 3 SD values was used as the cutoff value for seropositivity (J. Cook et al., unpub. data). A separate cutoff value was generated for each antigen (MSP-1_{19} and AMA-1) for each species (*P. vivax* and *P. falciparum*). The seroconversion rate was estimated by fitting a simple reversible catalytic model to the measured seroprevalence by age in years by using maximum-likelihood methods. The serologic-derived annual EIR was then estimated by using the MSP-1_{19} seroconversion rate and a calibration curve derived from determined values (*11*).The titer of antibody responses was estimated by using the formula dilution/[maximum OD/(OD test serum – minimum OD) – 1]; the median titer and interquartile range (IQR) are given. Because of low overall antibody prevalence, antibody responses were combined by species to determine the presence of any reactivity against *P. falciparum* or *P. vivax*. As a quantitative measure of reactivity to either malaria species, the highest titer in the MSP-1_{19} and AMA-1 ELISAs was used.

Factors associated with *P. falciparum* or *P. vivax* seroreactivity were determined for each village separately by using generalized estimating equations adjusting for correlation between observations from the same household. The following factors were tested in the models: age in years, distance to the nearest seasonal river (in 100 m), distance to the nearest enclosure of livestock (in 100 m), number of household members, number of houses in a 100-m radius, roofing material, wall material, floor material, travel history, recent or regular bed net use, and an indicator of household wealth. The household wealth index was calculated on the basis of principal component analysis on characteristics such as ownership of a television, radio, telephone, bicycle, motorbike, cattle, and access to electricity (*19*). Variables that were significant at $p = 0.10$ in univariate analyses were added to the multivariate model and retained in the final multivariate model if their association with immune responses was statistically significant at $p<0.05$.

For detection of spatial clusters in immune responses, age-adjusted $\log_{10}$-transformed ODs were calculated as described by Wilson et al (*20*). First, Loess lines were fitted to scatter plots of age against log-transformed ODs for each antigen separately. For *P. falciparum* MSP-1_{19} and *P. vivax* AMA-1, the linear regression was split at 49 and 46 years of age. Log-transformed ODs were adjusted for age by linear regression. SaTScan software (*21*) was used for detection of spatial clustering in log-transformed age-adjusted OD values by using the normal probability model. A circular-shaped window was used to systematically scan the area of each village separately; statistical significance of the clusters was explored by using 999 Monte Carlo replications to ensure adequate power for defining clusters. The upper limit was specified as 50% of the village population. Significant increases in ODs were detected by calculation of the likelihood ratio for each window. Only clusters were reported that appeared for MSP-1_{19}, AMA-1, and their combined age-adjusted ODs. Maps were made by using ArcGIS version 9.1 (Environmental Systems Research Institute).

Results

The 2 cross-sectional surveys were completed in March (dry season, n = 1,178) and August–September (wet season, n = 1,128) 2008. These surveys were characterized by a clear seasonality with no rainfall detected during November 2007–March 2008 and a median monthly rainfall of 114.5 mm in April–August 2008. None of the survey participants were positive by rapid diagnostic test, and *P. falciparum* or *P. vivax* parasites were not detected on any of the examined blood slides (Table 1). Available hospital records indicated 2/283 slide-confirmed, RDT-confirmed malaria cases in the study area in July and August 2008 (T. Bousema, unpub. data). Travel history was not available for these persons. During August–September 2008, a total of 464 potential breeding sites were examined in Xuunshaley (n = 40), Badahabo (n = 42), and Ceel-Bardaale (n = 382). In Ceel-Bardaale, 158 *Anopheles* mosquito larvae were found at 81 of 382 examined sites. In the 2 other villages, no *Anopheles* larvae were observed.

Malaria Exposure Assessed by Immunologic Methods

In August–September 2008, serum samples were collected from 1,128 persons in Xuunshaley (n = 271), Badahabo (n = 160), and Ceel-Bardaale (n = 697) (Table 2). In the 3 months before the survey, 19 persons reported having traveled to areas that are known to have higher malaria endemicity for a median of 4 (IQR 2–20) days. Persons who reported traveling to areas highly endemic for malaria were more likely to have a positive response to *P. falciparum* (odds ratio [OR] 2.62, 95% confidence interval [CI] 0.98–7.01, p = 0.054) but not to *P. vivax* (OR 1.18, 95% CI 0.42–3.32, p = 0.75), after adjustment for age and village of residence. These 19 persons were excluded from further analyses.

All antigens tested showed a clear increase in seroprevalence with a person's age (Figure 1). The data did not suggest a recent reduction in malaria transmission intensity (*14*). The EIR for *P. falciparum* based on seroconver-

Table 1. Characteristics of persons included in cross-sectional survey for *Plasmodium falciparum* and *P. vivax* infection, Somalia, 2008*

Characteristic	Dry season, Mar–Apr	End of wet season, Aug–Sep
No. persons	1,178	1,128
Age, y, median (IQR)	17 (6–36)	15 (6–37)
Female	48.6 (573/1,178)	50.8 (573/1,128)
Reported regular bed net use	1.9 (22/1,158)	2.2 (25/1,128)
Reported fever in 14 d preceding survey	4.8 (57/1,179)	0.6 (7/1,128)
Temperature ≥37.5°C at time of survey	0.8 (10/1,177)	1.1 (12/1,124)
Positive rapid diagnostic test result	0 (0/1,173)	0 (0/1,106)
Plasmodium falciparum parasite prevalence†	0 (0/1,173)	0 (0/1,106)
P. vivax parasite prevalence†	0 (0/1,173)	0 (0/1,106)

*IQR, interquartile range (25th–75th percentile). Values are % (no. positive/no. tested) unless otherwise indicated.
†Determined by screening 200 high-power microscopic fields.

sion rates for MSP-1_{19} and AMA-1 (*11*) was <0.1 infectious bites/person/year. When MSP-1_{19} and AMA-1 data were combined, 17.9% (179/1,001) of the persons tested showed reactivity against *P. falciparum* (i.e., had antibodies against *P. falciparum* MSP-1_{19}, AMA-1, or both) and 19.3% (202/1,044) against *P. vivax*. There was a significant positive association between reactivity against *P. falciparum* and *P. vivax* (p<0.001). However, only 39.8% (66/166) of persons with antibodies against *P. falciparum* also responded against *P. vivax* antigens, and there was no apparent correlation between antibody titers against antigens of the 2 malaria species (p≥0.58).

Spatial Patterns in Seroreactivity

P. falciparum antibody prevalence was 9.4% (23/244) in Xuunshaley, 21.7% (30/138) in Badahabo (p = 0.001), and 20.4% (126/619) in Ceel-Bardaale (p<0.001) (Table 2). *P. vivax* antibody prevalence was 16.1% (40/248) in Xuunshaley, 21.0% (31/148) in Badahabo (p = 0.11), and 20.2% (131/648) in Ceel-Bardaale (p = 0.13) (Table 2).

Age-adjusted *P. falciparum* seroreactivity was significantly increased in a cluster of 18 households (108 persons) in Ceel-Bardaale (p = 0.002) (Figure 2). In Xuunshaley, there was a small cluster of 6 households (27 persons) with a higher age-adjusted *P. vivax* seroreactivity (p = 0.005).

Factors Associated with Seroreactivity

Seroreactivity data were analyzed for villages separately because villages were ≥7 km apart and were therefore likely to have their own transmission characteristics. In all 3 villages, *P. falciparum* antibody prevalence increased with age (Table 3). For Ceel-Bardaale, an independent negative association was found between *P. falciparum* antibody responses and distance to the nearest seasonal river (OR 0.94, 95% CI 0.88–0.99, p = 0.03) after adjustment for age and correlation between observations from the same household. Within the group of persons who had a positive antibody response against *P. falciparum*, the titer increased with age in Xuunshaley (β = 1.74, SE = 0.81, p = 0.031) and Ceel-Bardaale (β = 11.48, SE = 3.49, p = 0.001).

Similar to *P. falciparum*, *P. vivax* antibody prevalence increased with age in all 3 villages (Table 3). For Ceel-Bardaale, distance to the nearest seasonal river was negatively associated with *P. vivax* immune response (OR 0.93,

Table 2. Immune responses against *Plasmodium falciparum* and *P. vivax* in study participants, by village, Somalia, 2008*

	Village†			
Characteristic	Xuunshaley	Badahabo	Ceel-Bardaale	p value‡
No. persons	271	160	697	
Median age, y (IQR)	20 (7–40)	17.5 (5–35)	13 (6–35)	0.04
P. falciparum immune response				
Combined	9.4 (23/244)	21.7 (30/138)	20.4 (126/619)	<0.001
MSP-1	5.1 (13/254)	13.4 (19/142)	15.0 (95/634)	<0.001
	251.2 (155.0–285.3)	214.8 (169.8–275.5)	248.5 (190.5–397.2)	0.12
AMA-1	4.8 (12/252)	9.2 (13/141)	8.9 (58/653)	0.02
	169.1 (137.0–190.9)	189.3 (158.4–225.5)	233.7 (170.7–487.0)	0.12
P. vivax immune response				
Combined	16.1 (40/248)	21.0 (31/148)	20.2 (131/648)	0.33
MSP-1	11.9 (30/252)	13.9 (21/151)	10.4 (58/648)	0.33
	333.9 (271.4–463.9)	342.4 (280.1–374.8)	291.5 (248.7–393.3)	0.39
AMA-1	5.2 (13/252)	7.4 (11/149)	12.8 (85/665)	0.001
	151.5 (146.0–202.1)	183.5 (155.7–275.7)	227.1 (184.6–390.8)	0.10

*IQR, interquartile range (25th–75th percentile); MSP-1, merozoite surface protein 1; AMA-1, apical membrane antigen 1.
†Values are % prevalence (no. positive/no. tested) or approximate median titer (IQR) only for seropositive persons unless otherwise indicated.
‡Adjusted for age and correlations between observations from the same household, when applicable.

Table 3. Factors associated with *Plasmodium falciparum* or *P. vivax* seroprevalence in 3 villages, Somalia, 2008*

		P. falciparum		*P. vivax*	
Village	Factor	OR (95% CI)	p value	OR (95% CI)	p value
Xuunshaley	Age	1.02 (1.00–1.04)	0.029	1.04 (1.02–1.06)	<0.001
Badahabo	Age	1.03 (1.01–1.05)	0.002	1.03 (1.01–1.05)	0.006
Ceel-Bardaale	Age	1.03 (1.02–1.04)	<0.001	1.03 (1.02–1.04)	<0.001
	Distance to river†	0.94 (0.88–0.99)	0.028	0.93 (0.88–0.99)	0.016

*OR, odds ratio; CI, confidence interval. Estimates are adjusted for correlation between observations from the same household.
†Nearest seasonal river.

95% CI 0.87–0.99, p = 0.02) after adjustment for age and correlation between observations from the same household. *P. vivax* antibody titer did not increase with age or any other factor in those persons who were seropositive. Household factors, socioeconomic factors, distance to the nearest livestock enclosure, and use of mosquito netting were not independently associated with immune responses against *P. falciparum* or *P. vivax*.

Although seroprevalence and antibody titers were higher in older age groups, seroreactivity was also observed in young children. *P. falciparum* antibodies were detected in 22 children ≤5 years of age (median titer 216.5, IQR 173.2–248.5); 10 had antibodies against *P. vivax* (median titer 220.1, IQR 190.4–262.4), and 2 of these children had antibodies against *P. falciparum* and *P. vivax*. Thirty children ≤5 years of age who responded to malaria antigens were from all 3 villages (3 from Xuunshaley, 7 from Badahabo, and 20 from Ceel-Bardaale). Travel to areas in which malaria was highly endemic in the past 3 months was not reported for any of these children with antibodies against *P. vivax*, *P. falciparum*, or both. In children ≤5 years of age, a response against *P. falciparum* antigens was not related to a response against *P. vivax* antigens (p = 0.30).

Discussion

We showed that serologic markers can be used to detect heterogeneity in malaria transmission in the Gebiley District of Somalia where malaria transmission occurs at levels too low to be detected by microscopy. None of the slides or rapid diagnostic tests showed parasite carriage in the population, and MSP-1_{19} and AMA-1 seroprevalence data showed a clear increase in seroreactivity with age and evidence for variation in exposure to malaria between and within villages.

Malaria is perceived as a public health problem in the study area (*22*), and the 2 slide-confirmed malaria cases confirm local clinical malaria episodes. Malaria transmission in the Gebiley District could not be confirmed by microscopy or RDT in 2 large cross-sectional surveys in the general population. However, our serologic findings confirmed the occurrence of malaria transmission in the area. Using a validated model to relate age-specific seroconversion rates to EIR (*11*), we estimated that *P. falciparum* transmission intensity in this area in Somalia was low (EIR <0.1 infectious bites/person/year). Because of the longevity of antibody responses, this estimate should be interpreted as an average EIR experienced over several years. The low EIR appeared to be supported by examination of breeding sites at the end of the wet season, which confirmed the presence of malaria vectors at a low density. We did not directly determine the EIR by sampling adult mosquitoes because the low density of mosquitoes would have required intensive sampling over different seasons (*6*,*23*).

Serologic data showed a clear age-dependency in malaria-specific immune responses, which suggested exposure-driven age acquisition of antibody response. Once acquired, antibody responses to MSP-1_{19} and AMA-1 will persist for several years (L.C. Okell, unpub. data) (*12*), and the rate of acquisition in younger age groups is therefore critical for determining current malaria transmission intensity. The maximum seroprevalence for individual malaria antigens did not exceed 25% in the oldest age groups, which is comparable to areas of low malaria endemicity in northeastern Tanzania (*12*).

Because of the longevity of antibody responses, seroreactivity may not necessarily be the result of recent exposure or exposure in the study area (*11*,*24*–*26*). Considerable changes in transmission intensity in the study area would have been detected by the model (*14*). However, especially in adults, exposure to parasites earlier in life and a history of traveling to malaria-endemic areas can obscure immune responses resulting from recent local transmission (*25*). Our data indicate that although antibodies may have been acquired outside the study area, ongoing local malaria transmission at a low intensity is likely. Elimination of false-positive results to reliably detect low-level local malaria transmission is necessary.

Cross-reactivity between immune responses to malaria and other parasites have been reported (*27*,*28*) but are expected to be more pronounced when whole parasite extract is used instead of recombinant proteins representing single antigens. The chance of cross-reactive antibody responses may be minimized by using sera at a minimum dilution of 1:80 (*27*). Our serum samples were tested at considerably higher dilutions and we observed no relation in antibody titers between the homologous antigens of *P. falciparum* and *P. vivax*. Moreover, our method for calculating seropositivity derives its seronegative population from within

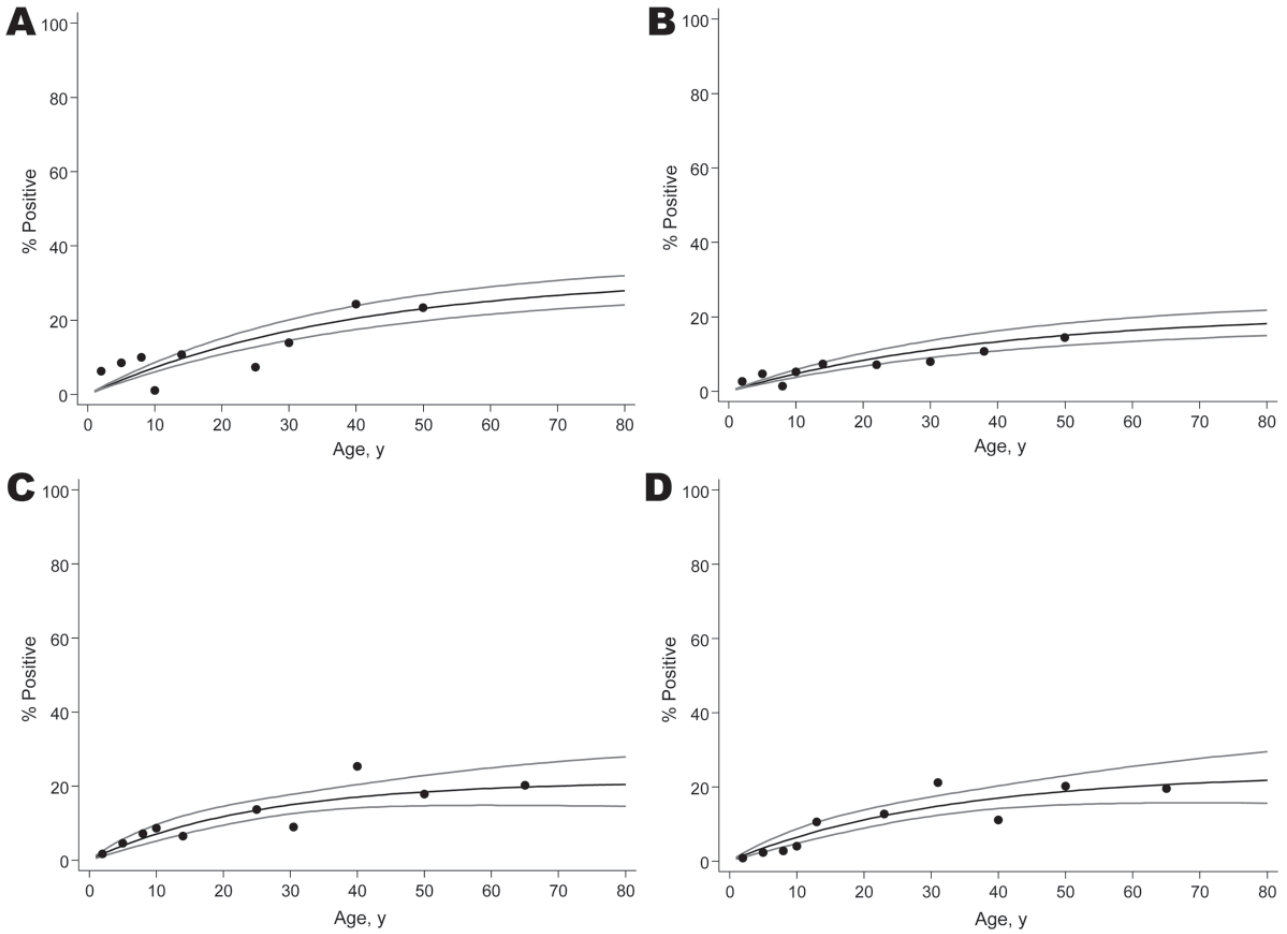

Figure 1. Seroprevalence data for antibodies against A) *Plasmodium falciparum* merozoite surface protein 1_{19} (MSP-1_{19}), B) *P. falciparum* apical membrane antigen 1 (AMA-1), C) *P. vivax* MSP-1_{19}, and D) *P. vivax* AMA-1 by age in the study population, Somalia, 2008. Gray lines indicate 95% confidence intervals. Seroconversion rates (95% confidence intervals) were as follows: *P. falciparum* MSP-1_{19} 0.0082 (0.0068–0.097); AMA-1 0.0053 (0.0042–0.0066); *P. vivax* MSP-1_{19} 0.0086 (0.0055–0.0133); AMA-1 0.0075 (0.0050–0.0112).

the study sample, thereby minimizing bias caused by local cross-reactive antigens. Although this method does not rule out cross-reactive antigens, it makes it unlikely. Antibody responses in young children who are unlikely to have acquired infections outside the study area, and for whom no recent travel history was reported, also suggest recent malaria transmission. In our study area, several children ≤5 years of age had antibody titers >200 to *P. falciparum* (n = 17) or *P. vivax* (n = 6). The presence of strong antibody responses (indirect fluorescent antibody titer ≥20) in children <15 years of age was used as evidence for active transmission of malaria in area of low endemicity in Middle America (Costa Rica) (*25*,*26*).

The indication for local malaria transmission we provide in this study is relevant for local health workers who should be prepared for fever investigations with standard parasitologic techniques (microscopy and RDT). Malaria should be considered as a plausible cause of febrile illness, particularly in an epidemic form. Low-intensity malaria transmission and the presence of malaria vectors make the area susceptible to malaria epidemics, which can have a high mortality rate in resource-poor areas (*29*), especially if outbreak detection systems (*30*) are not feasible because of a poor health infrastructure.

We observed heterogeneity in seroreactivity within the study area. Although the 3 villages had low transmission intensity and showed no difference in microscopic parasite carriage, serologic markers showed variation in malaria exposure. Antibody prevalence against *P. falciparum* and, less markedly, *P. vivax* were lowest in Xuunshaley, which was furthest from seasonal rivers. Combined *P. falciparum* MSP-1_{19} and AMA-1 antibody prevalence was 2× higher in Badahabo and Ceel-Bardaale than in Xuunshaley. SaTScan analysis indicated heterogeneity in malaria exposure at a microepidemiologic level. We observed 1 statistically significant cluster of persons with higher seroreactivity against

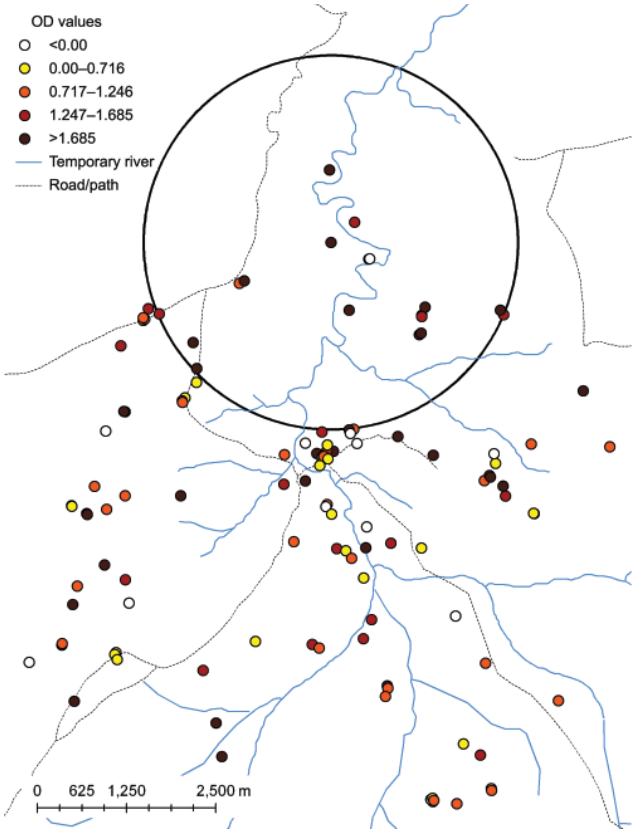

Figure 2. Age-adjusted optical density (OD) values for antibodies against *Plasmodium falciparum* in the study population, Ceel-Bardaale, Somalia, 2008. Colored dots indicate mean age-adjusted optical densities per household for combined seroreactivity to *P. falciparum* merozoite surface protein 1_{19} and apical membrane antigen 1. The large circle indicates a statistically significant cluster of higher *P. falciparum* seroreactivity that was detected by a spatial scan on the age-adjusted seroreactivity of individual study participants to both *P. falciparum* antigens (p = 0.002). As a result of age adjustment, some persons had lower than expected seroreactivities. This adjustment resulted in negative OD values.

P. falciparum and 1 with higher seroreactivity against *P. vivax*. In Ceel-Bardaale, where households were scattered along a delta of seasonal rivers, antibody prevalences to *P. falciparum* and *P. vivax* were negatively associated with distance to the nearest river. In several areas of higher endemicity, distance to the nearest body of water has been related to malaria incidence (*5*,*20*,*31*,*32*) and immune responses (*20*,*32*). No other factors were significantly related to malaria-specific immune responses.

Our data indicate that serologic markers can be used to determine variation in transmission intensity at levels of malaria transmission that are too low for sensitive assessments by microscopy, RDT, or entomologic tools. The sensitivity of serologic analysis to detect small-scale differences in transmission intensity may prove extremely useful in evaluating malaria control programs in areas where conventional malariometric markers fail. It may also provide vital information on which areas are most likely to be receptive to transmission if malaria epidemics were to occur.

Acknowledgments

We thank Abdikarim Yusuf for training and supervising of laboratory technicians; Fahim Yusuf and Ahmed Noor for facilitating training of surveyors and supervising field work; Tanya Shewchuk, the United Nations Children's Fund (UNICEF)–Somalia, the World Health Organization, UNICEF country offices, the Kenya Medical Research Institute (KEMRI), Wafaa Said, Ahmed Mohamed Jama, and Lydiah Mwangi for administrative support; Moses Mosobo and Ken Awuondo for technical support for microscopy; Bart Faber for providing *P. vivax* AMA-1 antigen; Shona Wilson, Patrick Corran, Lynn Spencer, Eleanor Riley, and Immo Kleinschmidt for support and advice; the director of KEMRI for support; and the clan elders, homestead heads, and community members for participating in the survey.

This study was supported by The Global Fund to Fight AIDS, Tuberculosis and Malaria to UNICEF–Somalia (YH/101/04/08). T.B. is supported by a Rubicon fellowship of the Netherlands Organisation for Scientific Research (825.05.025). J.C. (078925), C.D. (078925), A.M.N. (081829), and R.W.S. (079081) are supported by the Wellcome Trust UK.

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Infection of Kissing Bugs with *Trypanosoma cruzi*, Tucson, Arizona, USA

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Triatomine insects (Hemiptera: Reduviidae), commonly known as kissing bugs, are a potential health problem in the southwestern United States as possible vectors of *Trypanosoma cruzi*, the causative agent of Chagas disease. Although this disease has been traditionally restricted to Latin America, a small number of vector-transmitted autochthonous US cases have been reported. Because triatomine bugs and infected mammalian reservoirs are plentiful in southern Arizona, we collected triatomines inside or around human houses in Tucson and analyzed the insects using molecular techniques to determine whether they were infected with *T. cruzi*. We found that 41.5% of collected bugs (n = 164) were infected with *T. cruzi*, and that 63% of the collection sites (n = 22) yielded ≥1 infected specimens. Although many factors may contribute to the lack of reported cases in Arizona, these results indicate that the risk for infection in this region may be higher than previously thought.

Chagas disease is endemic throughout Mexico and Central and South America, with ≈7.7 million persons infected, 108.6 million persons considered at risk, 3–3.3 million symptomatic cases, an annual incidence of 42,500 cases (through vectorial transmission), and 21,000 deaths every year (*1*–*3*). This disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted to humans by blood-sucking insects of the family Reduviidae (Triatominae). Although mainly a vector-borne disease, Chagas disease also can be acquired by humans through blood transfusions and organ transplantation (*2*–*6*), congenitally (from a pregnant woman to her baby) (*7*), and through oral contamination, e.g., foodborne (*8*). Acute infection can be lethal, and cardiomyopathy develops in 25%–30% of infected persons (*1*). Although neither a vaccine against infection nor a completely effective treatment for chronic Chagas disease currently exists (*2*,*9*), treatment is now recommended for acute infections, congenital infections, infections in immunosupressed persons, and infections in children (*10*).

Although historically Chagas disease has been considered restricted to Latin America (*1*,*3*), the disease is becoming a serious health issue in the United States because of the presence of a notable number of blood donors seropositive for *T. cruzi* (*11*–*13*). Notably, a small number of the seropositive blood donors have never left the United States. Only 7 autochthonous cases of this disease have been reported in the United States, all in the southern half of the country (*14*–*19*). The most recent reported case of autochthonous transmission of *T. cruzi* occurred in 2006 near New Orleans, Louisiana (*18*). Many cases of Chagas disease in the United States, however, may be overlooked because the early phase of the infection is often asymptomatic (*9*,*16*), and health professionals are largely unaware of this disease. In Arizona, humans may be at a greater risk for vectorial transmission of the disease than previously thought because human populations are rapidly expanding into habitats where infected triatomines (*20*–*22*) and wild mammalian reservoirs such as packrats, mice, armadillos, raccoons, and opossums (*23*–*27*) are plentiful. Chagas disease is actively transmitted in domestic cycles involving dogs in southern Texas (*20*,*28*), where >50% of triatomines collected inside or near the homes of persons were found to be infected with

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DOI: 10.3201/eid1603.090648

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T. cruzi (*19*,*20*). Studies conducted many decades ago found that triatomines in California, Arizona, and New Mexico were also infected with *T. cruzi* (*22–25*,*29*).

Arizona is noteworthy as the state with the highest number of triatomine–human contacts reported in the United States (American Association of Poison Control Centers, www.aapcc.org/DNN; Arizona Poison and Drug Information Center, University of Arizona Health Sciences Center, www.pharmacy.arizona.edu/outreach/poison). In southern Arizona, triatomine bugs live in association mostly with the white-throated woodrat (*Neotoma albigula*) (*24*,*26*). Triatomine bugs have wingless nymphal stages and winged adults. During their dispersal season (beginning of May through July), adult insects, attracted by light, reach human habitations (*30–32*). *Triatoma rubida* is by far the most common species (Figure 1), but *T. protracta* and *T. recurva* are also found (*30*,*32*). *T. rubida* was associated with a clinical case of Chagas disease in the city of Guaymas, Mexico, although this bug is perhaps a different subspecies than the one found in Arizona (*33*).

To our knowledge, the most recent comprehensive studies about the infection rates by *T. cruzi* in triatomines from Arizona were conducted >45 years ago (*21*,*22*), by using microscopy to detect the presence of live parasites in the insect's gut or feces. In 1943, Wood (*22*) found an overall infection rate of 4% in triatomines (28 of 699) from Arizona collected over a 3-year period. In 1964, Bice (*21*) collected triatomines from packrat dens in what is today a densely populated area in metropolitan Tucson, Arizona, and found that 7.5% and 19.5% of *T. rubida* and *T. protracta* bugs, respectively, were infected with *T. cruzi* (*21*). A recent study that used molecular methods, but was based on a small sample, found that 1 in 4 *T. protracta* and 0 of the 20 *T. rubida* bugs examined were infected with *T. cruzi* (*34*).

To estimate the current potential of vectorial transmission of *T. cruzi* disease in southern Arizona, we investigated the infection rate of triatomines collected inside and around houses in metropolitan Tucson (Pima County), Arizona. Tucson is the second largest metropolitan area in Arizona with a population (as of 2007) of 1,003,235, of which 462,103 persons live in areas where triatomines are plentiful (*35*).

Materials and Methods

Collection of Insects

Triatomine insects were obtained by issuing public requests asking residents of metropolitan Tucson (32°13′18″N, 110°55′35″W), Arizona, to collect bugs found inside or around their houses. Insects that reach houses, as opposed to those directly collected from nests of wild animals, are of greatest epidemiologic importance because they have the highest chance of contact with humans. Collectors were instructed to use a container and not to touch or handle the insects with their bare hands, and they were usually informed about the way that Chagas disease is transmitted. In a preliminary study conducted in 2005, we found that some triatomine bugs were infected with *T. cruzi* (C.E. Reisenman et al., unpub. data). We therefore conducted a more extensive study in 2006. For each bug, we recorded, whenever possible, the collection site (address), insect species, stage, sex (if adults), and date of collection as well as any other information the collector provided. Collected insects were individually placed in 95% ethanol immediately after collection or upon death and stored at 4°C until analysis. Insects were collected during May 15–December 18, 2006.

Figure 1. Adult female kissing bug of the species *Triatoma rubida*, the most abundant triatomine species in southern Arizona. Scale bar = 1 cm.

Analysis of *T. cruzi*

Each insect was analyzed by PCR for the presence of *T. cruzi*. Before analysis the insect was removed from ethanol and dried overnight in a petri dish to remove traces of ethanol before DNA extraction. The lower abdomen of each bug was detached with a sterile razor blade and homogenized with a ceramic ball, or placed in a 1.5-mL microfuge tube with phosphate-buffered saline (≤80 μL) and homogenized with a hand-held mortar.

DNA was extracted following the instructions provided with the QiaAmp DNA Blood Mini Kit (QIAGEN 51106; QIAGEN, Valencia, CA, USA). The DNA was amplified by

PCR according to an established *T. cruzi* sample-processing protocol (*36*) by using the *T. cruzi*–specific primers TCZ1 (5′-CGAGCTCTTGCCCACACGGGTGCT-3′) and TCZ2 (5′-CCTCCAAGCAGCGGATAGTTCAGG-3′), which amplify 188 bp of a repetitive nuclear sequence (*15*). For the minicircle locus, DNA was amplified by using primers S35 (5′-AAATAATGTACGGGKGAGATGCATGA-3′) and S36 (5′-GGGTTCGATTGGGGTTGGTGT-3′) (*37*), which amplify a 330-bp minicircle sequence. A 50-μL reaction containing 0.4 μM of each primer, 20–40 ng of template DNA, and DNA polymerase (GoTaq; Promega, Madison, WI, USA, or Platinum Taq; Invitrogen, Carlsbad, CA, USA) was prepared. Primers for PCR were made at the Centers for Disease Control and Prevention (Atlanta, GA, USA) core facility or acquired from Invitrogen. The cycling parameters for the reactions with the TCZ1 and TCZ2 primers were as described (*36*). The cycling parameters for the reactions that used the S35 and S36 primers were an initial denaturation at 95°C for 10 min, 35 cycles of amplification at 95°C (30 s each), 58°C (30 s each) and 72°C (1 min each), and a final extension at 72°C for 10 min. Samples were processed in a Mastercycler Gradient Thermocycler Machine (Eppendorf, Hauppauge, NY, USA) or an iCycler (Bio-Rad, Hercules, CA, USA). PCR products were subjected to electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized by using UV transillumination with AlphaImager program (Alpha Innotech, San Leandro, CA, USA). All PCRs were run with a positive control of known *T. cruzi* DNA and with a negative control in which template DNA was omitted. Results that were positive for both sets of primers were considered positive. If a sample was positive for only 1 set of primers, then the products of the PCR were cloned (pGem-T Easy Vector System; Promega) and sequenced (Big Dye Terminator, v1.1 and ABI 31 30xl Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). Cloned sequences were compared with sequences in GenBank to determine if the amplified sequence belonged to the *T. cruzi* genome. A random sample of ≈15% negative samples (n = 11) was analyzed along with positive samples to exclude the possibility of false-negative samples.

Results

Insect Collection and Demographics

A total of 164 triatomine bugs (158 [96.3%] *T. rubida*, 5 [3%] *T. recurva*, and 1 [0.6%] *T. protracta*) were collected by volunteers and analyzed for *T. cruzi*. Most of the collected *T. rubida* were adults (93.6%, n = 151). Of the 141 adult *T. rubida* identified by sex, 87 were females (62%) and 54 were males (38%). The proportion of females to males was statistically different from a 1:1 sex ratio (χ^2 = 8.2, df = 1, p = 0.004).

Twenty-two collectors provided a total of 142 insects, with each collector contributing a variable number of insects per night (range 1–10, median 2). A single collector provided 73 insects collected on 16 nights throughout the dispersal season. Twenty-two additional bugs were collected by an unknown number of anonymous persons. Information about the specific location where insects were collected was obtained for 84% (n = 139 insects provided by 19 collectors) of the insects. These 139 insects (all *T. rubida*) were obtained from 17 collection sites distributed in 6 of the 8 metropolitan Tucson areas corresponding to the cardinal and ordinal points of the compass, and from 2 collection sites in central Tucson (Table). Because insects were collected by volunteers rather than by using systematic collection methods (i.e., light traps set up in all geographic areas), the information in the Table serves the sole purpose of reporting where insects were collected and does not constitute an estimate of the abundance of insects per area.

Adult *T. rubida* insects were collected in or around houses from mid-May through the end of August (Figure 2). Most adults were collected in the last days of May and first week of June (Figure 2, panel B); a total of 61% of insects were caught during May 25–June 8. This peak in insect collections coincides with a typical, sustained increase in minimum temperatures that enables insects to fly at night (*32*) (Figure 2, panel A). Bugs were collected steadily throughout the last week of June; only 13 adults (8%) were collected during the rest of the dispersal season, which extends to the end of August. Although insects were not collected by using systematic methods, peak collection periods coincide with the peak dispersals reported by Ekkens (*32*).

Analysis of Infection by *T. cruzi*

We found that 68 (41.5%) of the 164 bugs collected were infected with *T. cruzi*. Twenty-four (35%) of the samples were positive by both set of primers and therefore

Table. Collection sites and collected insects per area, triatomine insects survey, metropolitan Tucson, Arizona, USA, 2006*

Area	No. collection sites (% with insects infected with *Trypanosoma cruzi*)	No. insects collected (% infected with *T. cruzi*)
Central	2 (100)	2 (100)
North	1 (0)	2 (0)
Northeast	1 (100)	2 (50)
Northwest	6 (66)	14 (43)
South	0	0
Southeast	3 (66)	11 (45)
Southwest	2 (100)	19 (42)
East	0	0
West	4 (100)	88 (40)

*Information about collection sites was obtained for 139 of the 164 bugs collected. An individual collector from the western area provided an unusually large number of insects (n = 73).

were considered positive. The remaining 44 (65%) positive samples were positive for S35/S36 only, but all of them were confirmed positive by cloning and sequencing, thus excluding the possibility of false-positive results. No samples were positive for TCZ1/TCZ2 and negative for S35/S36.

Of the 22 identified sites or houses where insects were collected, 14 (63%) had at least 1 bug infected with *T. cruzi*. Infected bugs were found in 7 of 8 areas, including central Tucson (Table). The percentage of infected bugs per area was variable (median 43%, range 0%–100%), likely due to the low number of bugs (1–2) collected in certain areas (e.g., central, north, northeast). The mean ± SD percentage of infected bugs per area, considering only those areas where >10 insects were collected, was 42.5% ± 1.0% (4 geographic areas, n = 132 insects). Similarly, to estimate the prevalence of infection per collection site, we selected sites where at least 5 bugs were collected. The mean ± SD number of infected bugs per collection site was 47.2% ± 5.7% (n = 7 collection sites in 4 geographic areas, n = 120 insects). This percentage was slightly higher (48.8 ± 6.6%, n = 6 collection sites) when a site where a large number of bugs were collected (n = 73) was excluded from the analysis.

The prevalence of infection by *T. cruzi* among triatomine species was variable, as reported (*21*), although a larger sample is necessary to confirm this prevalence. Forty-one percent of *T. rubida* (n = 158) bugs, 60% of *T. recurva* (n = 5) bugs, and the single *T. protracta* bug collected were infected with *T. cruzi*. Because only a few *T. recurva* and *T. protracta* bugs were collected, we restricted all further analysis to *T. rubida*. Forty-two percent of nymphs (n = 7), 40.1% of females (n = 87), and 40.0% of males (n = 54) of *T. rubida* were found to be infected with *T. cruzi*. Among adults, the probability of infection was independent of sex ($\chi^2 = 0.015$, df = 1, p>0.9, by χ^2 contingency analysis). Infected bugs were found throughout the year; the median number of infected insects per 5-day collection period during the dispersion season (mid-May through mid-July) was 27% (range 17%–67%).

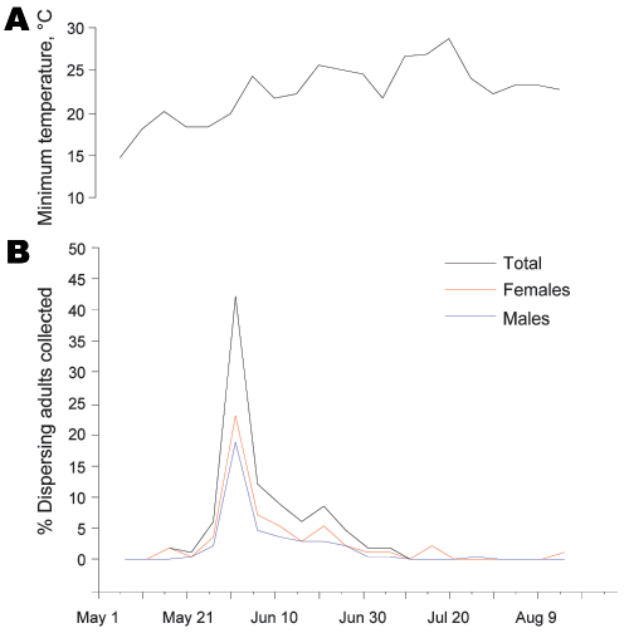

Figure 2. Temporal pattern of adult *Triatoma rubida* insects collected in metropolitan Tucson, Arizona, USA, May–August, 2006. A) Average minimum daily temperature recorded in 2006 during the period shown (data obtained from www.wrh.noaa.gov/twc/climate/reports.php). B) Percentages of all adults (n = 134), males (n = 52), and females (n = 82) collected during the period, in 5-day intervals (e.g., the percentage of insects collected during May 15–19 is represented on May 17). Information about sex or collection date was not available for 16 adults, so they were not included in this plot.

Discussion

To our knowledge, almost no information has been collected during the last half-century on the incidence of infection by *T. cruzi* in triatomine bugs from Arizona (but see below). We found that 41.5% of the 164 collected bugs, most of which were *T. rubida*, were infected with *T. cruzi*, and that 63% of houses or sites where insects were collected had at least 1 specimen infected. Most bugs collected were adults, and this winged life stage is known to be the main driver of dispersal (*38*). Although most bugs were collected inside or around human houses from May through the end of June, infected bugs were collected throughout the period of study. Specimens of the less abundant species *T. recurva* and *T. protracta* were also found to be infected. Samples that were positive with only 1 set of primers were confirmed by sequencing of the amplified DNA, excluding the possibility of false-positive results. In contrast with our results and previous research by others (*21*,*22*), a recent study found that none of the *T. rubida* bugs collected in the Tucson area were infected with *T. cruzi* (*34*). This discrepancy might be explained by the use of a different set of primers, the low numbers of insects examined (n = 20 in the aforementioned study), or bias in the insect sample, such as few collection sites. Furthermore, the infection rate reported here is much higher than that reported in earlier studies in Arizona, which ranged from 4% to 9% (*22*,*24*,*29*). Those studies were conducted by using microscopy that visualized the presence of the parasite in the insect gut; therefore, discrepancies maybe be attributed to differences in the sensitivity of the methods used (e.g., *16*).

The infection rates reported in this study, however, are in line with those reported in other recent systematic studies. For instance, 51% of triatomines (mostly *T. gerstaeckeri*) collected from several areas in Texas were infected

(n = 241), with many insects found near human dwellings (*19*). In Guaymas, in northwestern Mexico, 81% of *T. rubida* collected in houses and in the peridomicile (n = 279) were infected with *T. cruzi* (*39*). The fact that in that region adults and juveniles of *T. rubida* were found inside houses indicates a progressive domiciliation of this otherwise wild species, probably related to housing developments in triatomine habitats (*39*). In our study, immature stage (nymphs) insects collected inside houses were also infected, but the numbers are too small to draw any definitive conclusions. If these houses are sites of bug colonization, then the risk for human infection may be higher than in houses where only adult insects were found and removed. Nevertheless, because most immature insects in our study were found 1–4 months after the peak of dispersion (i.e., they are likely the offspring of adults that invade houses earlier) rather than consistently throughout the year, *T. rubida* bugs do not appear to be in the process of becoming domiciliated in Arizona.

Why have there been no reports of autochthonous cases of Chagas disease in Arizona despite our finding that 41.5% of bugs are infected with *T. cruzi*? In southern Arizona, triatomines live in close association with the sylvatic animal reservoirs upon which they feed (*26*) and apparently have a low capacity for domiciliation, although juvenile insects (the offspring of dispersing adults) can be found in houses near beds and readily feed on humans if necessary. Good housing conditions (e.g., lack of crevices in walls or ceilings) do not favor the permanent domiciliation of the insects, but this may not be the case in rural areas where housing materials provide shelter for the insects. Under those circumstances, colonization of human habitats might be favored because at least half of dispersing adults were female and likely gravid (C.E. Reisenman, unpub. data). In principle, the parasite can be transmitted to humans when infected insects that invade houses defecate on the skin of a human host upon feeding. Although a recent study reported that *T. rubida* and *T. protracta* do not defecate while feeding (*34*), our current investigations indicate that this is not the case for *T. rubida* bugs in all stages and for both sexes (C.E. Reisenman, unpub. data). Pet dogs can become infected by contamination with excreta but also by contact with the oral mucosa when they instinctively chew insects that might be infected (*40*).

Other reasons that might explain why Chagas disease is so rare in the United States are the following: misdiagnosis of the early infection (*9*,*16*), low insect vectorial capacity (*34*), or low infectivity of the genetic lineage of the *T. cruzi* parasites present in local insects and mammals, although this remains to be investigated. Bice (*21*) showed the presence of *T. cruzi* parasites in the heart muscle of a mouse inoculated with feces from an adult *T. rubida* bug collected in the Tucson area. Should the lineage of *T. cruzi* present in southern Arizona correspond to that associated with the pathogenic form of Chagas disease, the data presented here suggest that vectorial transmission of the disease in the area is possible.

Acknowledgments

We thank the 22 volunteer collectors for providing insects, especially Phil Jenkins, Bill Savary, Jillian Savary, Robert Smith, and Carl Olson. We also thank the Coordinating Center for Infectious Diseases Core Facility at the Centers for Disease Control and Prevention and C. Olson for identifying insects, Andrew Dacks for critically reading this manuscript, and members of the Hildebrand laboratory for helpful discussions.

This work was supported by an Arizona Biomedical Research Commission grant no. 0708 (to J.G.H.).

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Surveillance for West Nile Virus in American White Pelicans, Montana, USA, 2006–2007

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West Nile virus (WNV)–associated deaths of American white pelican (*Pelecanus erythrorhynchos*) chicks have been recognized at various nesting colonies in the United States since 2002. We evaluated American white pelican nesting colonies in Sheridan County, Montana, USA, for an association between WNV-positive pelican carcasses and human West Nile neuroinvasive disease. Persons in counties hosting affected colonies had a 5× higher risk for disease than those in counties with unaffected colonies. We also investigated WNV infection and blood meal source among mosquitoes and pelican tissue type for greatest WNV detection efficacy in carcasses. WNV-infected *Culex tarsalis* mosquitoes were detected and blood-engorged *Cx. tarsalis* contained pelican DNA. Viral loads and detection consistency among pelican tissues were greatest in feather pulp, brain, heart, and skin. Given the risks posed to wildlife and human health, coordinated efforts among wildlife and public health authorities to monitor these pelican colonies for WNV activity are potentially useful.

After West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) was detected in the Great Plains of the United States in 2002, programs were initiated to identify the spatial distribution of WNV transmission risk throughout the region. Surveillance activities included compiling case counts for human and equine disease, and testing mosquitoes, avian carcasses, and sentinel chicken serum samples for WNV infection. Corvid (primarily crows and magpies) death surveillance was an effective early warning system for human disease shortly after WNV was detected in this region (*1*). However, carcasses of numerous other bird species also were positive for WNV (*2*). Avian deaths caused by WNV infection typically result in widely dispersed carcasses; for the extent of these deaths to be recognized, substantial public cooperation is required in reporting deaths (*3*). In contrast to this cryptic pattern of deaths, geographically focused deaths among juvenile American white pelicans (*Pelecanus erythrorhynchos* Gmelin; order Pelecaniformes, family Pelecanidae) have occurred as a result of WNV transmission at numerous pelican-breeding colonies throughout the northern Great Plains (*4*). This region of the United States has the highest incidence of human West Nile neuroinvasive disease (WNND) recorded (*5*).

Concurrent with the arrival of WNV to the northern Great Plains region, high death rates of pelican chicks were observed at 4 major colonies in Montana, North Dakota, South Dakota, and Minnesota. WNV was presumed to be the etiologic agent for >9,000 American white pelican deaths in 7 states in 2002–2003 on the basis of testing of a sample of carcasses from various affected colonies (*6*). At Medicine Lake National Wildlife Refuge (MLNWR) in Montana, the chick death rate from mid-July until fledging, a time when pelican chicks are less vulnerable to severe weather and predation, typically averages ≤4%. However, this death rate reached as high as 44% among colonies in the region after the arrival of WNV in 2002, and annual losses since then have remained elevated (typically 7–8×) in most years (*4*). Although a spatiotemporal link between WNV detection and pelican chick deaths seems evident, the cause of most of these deaths remains presumptive. Furthermore, the potential public health consequences of American white pelican deaths need to be evaluated.

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DOI: 10.3201/eid1603.090559

Pelican deaths may indicate increased risk for WNV transmission to persons living in nearby communities. We evaluated pelican deaths and human WNND cases for potential associations. In addition, we captured and tested mosquitoes from MLNWR in 2006 and 2007 to determine the risk for vector-borne transmission of WNV and identify the vertebrate source of mosquito blood meals. Finally, we collected a series of tissue types from a subset of pelican carcasses at our field site to identify the most efficient tissue for maximizing the probability of WNV detection and to confirm WNV infection as a contributing factor to elevated prefledgling pelican death rates.

Materials and Methods

Site Description

MLNWR (elevation 590 m) is located in Sheridan County in northeastern Montana (48°27′N, 104°23′W). The refuge covers 13,000 hectares, including Medicine Lake (3,320 hectares), the largest natural lake in eastern Montana. Extensive wetlands provide suitable breeding habitat for mosquitoes and aquatic birds. Cropland and short-grass prairie surround the lake and provide nesting grounds to ≈125 species of birds. Approximately 4,000 breeding pairs of pelicans nest on a narrow peninsula (length ≈500 m) (*7*).

Spatiotemporal Associations between Pelican Deaths and Human WNV Disease

Data for human WNV disease cases were obtained from ArboNET, an internet-based passive surveillance system maintained by the Centers for Disease Control and Prevention (Fort Collins, CO, USA) in collaboration with state and local health departments. Locations of colonies were obtained from King and Anderson (*8*). Wildlife Mortality Quarterly Reports published by the US Geological Survey National Wildlife Health Center provided locations and dates of WNV-related pelican deaths during 2003–2007 (www.nwhc.usgs.gov/publications/quarterly_reports/index.jsp). We used an odds ratio to compare incidence of WNND cases in counties with reported WNV-associated deaths at pelican-nesting colonies to that in all other counties with pelican colonies during 2003–2007. Positive and negative predictive values were defined as the percentage of counties with pelican-nesting colonies in which a pelican WNV die-off and human WNND case(s) occurred, and in which neither a pelican WNV die-off nor a human WNND case occurred, respectively, during a given year.

Although neuroinvasive and nonneuroinvasive disease cases are reportable, reporting of nonneuroinvasive WNV disease has varied substantially by jurisdiction and over time. Therefore, only WNND cases were considered. For each year and county that pelican and human WNV disease were observed, we determined the interval between the earliest collection date of WNV-positive pelicans and the earliest onset date of human WNV disease.

Surveillance of Mosquitoes

Mosquitoes were collected from MLNWR by using battery-powered miniature light traps (J.W. Hock, Gainesville, FL, USA) supplemented with CO_2 from a 9-kg compressed gas tank. In 2006, seven traps were placed at 5 locations on the northeast perimeter of Medicine Lake; 3 were at Bridgerman Point, 10–200 m from pelican-nesting and -congregation sites (*9*). In 2007, all 5 traps were placed at Bridgerman Point. Traps were generally operated for 2 consecutive nights each week from mid-May through August in 2006 and 2007, and collections were stored at –20°C for ≥24 h before transport on dry ice. Collections were processed on a chill table and the light trap index (LTI) was calculated for each week as the number of trapped *Culex tarsalis* mosquitoes per trap night.

For WNV testing by reverse transcription–PCR (RT-PCR), weekly trap collections were sorted by species and location. Pools of ≤50 adult female mosquitoes were homogenized in vials containing 4.5 mm-diameter copper-coated steel beads (BB pellets) in BA-1 medium (medium 199 with Hanks balanced salt solution, 0.05 mol/L Tris buffer, pH 7.6, 1% bovine serum albumin, 0.35 g/L of NaHCO3, 100 mg/L streptomycin, 100 U/mL penicillin G, 1 μg/mL amphotericin B) and clarified by centrifugation. RNA was extracted from the supernatant and purified through an EasyMag extractor (bioMérieux, Durham, NC, USA) by using automated magnetic silicon extraction. Purified RNA was transcribed into cDNA and amplified by using specific WNV primers as described (*10*) in an EasyQ thermocycler (bioMérieux). Detection of WNV cDNA was achieved by agarose gel electrophoresis. Prevalence of WNV infection in mosquito populations was estimated by using PooledInfRate (www.cdc.gov/ncidod/dvbid/westnile/software.html). Vector index was calculated as the product of the LTI and infection rate (*11*).

Identification of Mosquito Blood Meals

Blood-engorged *Aedes vexans* and *Cx. tarsalis* mosquitoes were stored individually at –20°C and processed for blood meal identification as described (*12*). Briefly, each mosquito was homogenized and DNA was extracted. A portion of the mitochondrial cytochrome B gene was amplified and sequenced, and the resulting sequence was compared with sequences in a database for species identification.

Pelican Sample Collection and Preparation

Moribund pelican chicks (≈6–12 weeks of age) showing clinical signs suggestive of WNV infection (e.g., ataxia, torticollis, reluctance or inability to move) (Figure) were killed by cervical dislocation and stored frozen until

Figure. Juvenile American white pelicans (*Pelecanus erythrorhynchos*) at Medicine Lake National Wildlife Refuge, Montana, USA, 2007, including ill (foreground) and dead (background) birds.

necropsy. In 2006, carcasses were collected from July 25 through August 11 (n = 8) during a period of maximum chick deaths, and in 2007 from July 11 through August 1 (n = 24) after confirmation of WNV in mosquito pools. Also in 2007, oropharyngeal and cloacal swab samples, skin, and feather samples were collected from 23 carcasses in the field before they were frozen for comparison with samples collected from the same animals in the laboratory during necropsy.

For swab samples, dacron-tipped applicators were inserted into the oropharyngeal cavity (behind the pouch) or into the cloaca and then submerged and swirled in vials containing 1 mL BA-1 medium and discarded. In 2007, eye swab samples were collected from 17 carcasses by placing the applicator tip between the inner membrane of the eyelid and the eye. In addition, pouch lice (*Piagetiella peralis*) were individually removed from the inner lining of the pouch of each pelican and pooled in cryovials (≤40 lice/pool). Four flight feathers were removed from each carcass (2/wing). Feathers were removed from the follicle, and the calamus (quill tip) was aseptically cut and placed with the associated pulp into a vial containing 1 mL BA-1 medium. Approximately 0.5 cm^3 each of skin, kidney, spleen, heart, lung, and brain was aseptically collected and placed in cryovials containing 1 mL BA-1 medium for cryopreservation.

Tissues and chewing lice, obtained from the inside of throat pouches, were homogenized in a mixer mill (5 min at 25 cycles/s; Retsch GmbH, Haan, Germany) in 1 mL of BA-1 medium containing 20% fetal bovine serum and a BB pellet. Homogenates were clarified by centrifugation (12,000 × *g* for 3 min), and an aliquot was removed for immediate testing. Remaining supernatants were stored at –80°C.

Detection of WNV in Pelican Samples

Virus isolation was performed for tissues, lice homogenates, and swabs by using a Vero cell plaque assay as described (*13*). Viral plaques were confirmed as WNV by RT-PCR or VecTest WNV Antigen Detection Assay (Medical Analysis Systems, Camarillo, CA, USA) as described (*2*). RT-PCR and plaque assay detection methods were compared within tissue types by using the Fisher exact test with Bonferroni correction for 9 comparisons ($\alpha = 0.0056$). For specimens collected in the field and their carcass-matched controls collected in the laboratory, test results were compared by using the κ statistic for concordance.

RT-PCR methods for detection of WNV RNA in tissues were according to those of Lanciotti et al. (*14*), except for use of the Viral RNA Minikit (QIAGEN, Valencia, CA, USA) for RNA extraction and the Bio-Rad Icycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) for cDNA amplification. A cycle threshold value ≤37 was considered positive for target sequence amplification. Samples were screened with 1 pair of primers (genome positions were 10668 for forward primer, 10770 for reverse primer, and 10691 for probes) and positive results were confirmed with a second pair of primers (genome positions were 1160 for forward primer, 1229 for reverse primer, and 1186 for probe).

Results

Association of Pelican Deaths and Human WNV Disease

The probability of human WNND cases in counties with pelican nesting colonies increased 5× when WNV-associated deaths occurred among the pelicans (odds ratio 5.0, 95% confidence interval 1.9–13.0, n = 135 county-years). The positive predictive value of pelican deaths for human WNND cases was 55%, and negative predictive value was 81%. Pelican deaths were observed an average of 23.1 days (median 13.5 days) before human case onset and occurred before human disease onset in 12 (75%) of 16 county-years (Table 1).

Mosquito Surveillance

In 2006, a total of 414 *Cx. tarsalis* mosquitoes were captured in 67 light trap-nights from July 1 through August 5. Weekly LTI values for *Cx. tarsalis* mosquitoes ranged from 4.1 to 9.0 during July and early August (Table 2) when mosquito populations were low because of severe drought (larval production sites were dry or contained ephemeral water). None of 12 pools of *Cx. tarsalis* mosquitoes assayed were positive for WNV RNA.

In 2007, a total of 25,291 *Cx. tarsalis* mosquitoes were captured in 42 light trap-nights from June 30 through August 8 (Table 2). Weekly LTI values during this period

Table 1. Deaths in pelicans infected by WNV and WNV human disease in counties with nesting American white pelican colonies, United States, 2003–2007*

Year	County, state	Earliest date of pelican death	Earliest date of disease in humans	Difference, d
2003	Big Stone, MN	Jul 1	Aug 25	56
	Phillips, MT	Aug 1	Aug 15	14
	Sheridan, MT	Jul 23	Aug 1	9
	Stutsman, ND	Jul 15	Jul 26	11
	Day, SD	Jul 28	Jul 14	–14
2004	Big Stone, MN	May 30	Sep 1	93
2005	Sheridan, MT	Jun 23	Jul 14	52
	Stutsman, ND	Jun 17	Jul 18	31
	Day, SD	Jul 6	Aug 26	51
2006	Big Stone, MN	Jun 15	Jul 27	42
	Washoe, NV	Jul 14	Jul 10	–4
	Stutsman, ND	Jul 24	Aug 1	8
	Day, SD	Jul 19	Aug 1	13
	Brown, WI	Jul 15	Aug 10	26
2007	Stutsman, ND	Jul 7	Jul 1	–6
	Day, SD	Jul 9	Jun 27	–12

*WNV, West Nile virus.

ranged from 169 to 1,643. WNV was detected in 28 (32.2%) of 87 mosquito pools by RT-PCR, with the first positive mosquito samples collected during the week of July 8, 2007. WNV was detected in 10 of 20 pools of mosquitoes collected during the last week of July; we observed an estimated infection rate of 10.7/1,000 mosquitoes.

Vertebrate DNA sequences were obtained from blood-engorged abdomens of 22 mosquitoes collected in 2007, including 8 *Ae. vexans* and 14 *Cx. tarsalis*. All 22 mosquitoes had fed on American white pelicans.

WNV in Pelican Samples

Twenty-seven (84.4%) of 32 pelicans sampled had ≥1 tissues positive for WNV by plaque assay compared with 7 (87.5%) of 8 positive by RT-PCR (Table 3). Pelicans with WNV-positive tissues were collected from July 25 through August 11, 2006, and July 11 through August 1, 2007. Because pelicans were not collected before these dates, the timing of initial onset of WNV outbreaks in pelicans is unknown.

Skin was the most efficacious tissue for WNV detection in pelican carcasses. Viral loads were greatest in feather pulp, brain, heart, and skin. RT-PCR and plaque assay results were similar; detection rates did not differ among specific tissues or between field-collected vs. laboratory-derived samples (Table 4). Concordance (i.e., test agreement) was 82% ($\kappa = 0.82$) among matched field and laboratory samples from the same carcasses. All pouch lice samples were negative for WNV.

Discussion

We observed an association between human cases of WNND and WNV-induced juvenile pelican deaths in counties with pelican-nesting colonies. The positive and negative predictive values of pelican WNV-associated deaths for human WNND cases were similar in magnitude to those of American crow (*Corvus brachyrhynchos*) deaths. These findings suggest that monitoring of pelican deaths in colonies near human populations could be of potential use in public health–oriented WNV surveillance programs, many of which use crow deaths as indicators of local WNV activity and human risk (*15*).

Surveillance of pelican colonies for WNV activity could assist in presaging human WNV infection and associated disease. Pelican deaths were generally detected ≥2 weeks before WNV disease onset in humans. However, our observations were limited by numerous assumptions inherent to surveillance data, such as that human case-patients were infected in their home counties, that all human residents of each county were equally at risk for WNV infection, and that only residents of a county with a colony were potentially at risk. Because human settlements nearest a colony may pertain to a different county, a more accurate analysis would evaluate distance from the nesting colony as a risk factor for human cases, independent of county lines.

Table 2. *Culex tarsalis* mosquito infection data for WNV calculated weekly during 2 WNV transmission seasons, Medicine Lake National Wildlife Refuge, Montana, USA*

	2006			2007				
Week of collection	Light trap nights	Light trap index ± SD†	No. positive pools/no. tested	Light trap nights	Light trap index ± SD†	No. positive pools/no. tested	Infection rate‡ (95% CI)	Vector index§ (95% CI)
Jul 1–7	13	4.1 ± 4.3	0/17	4	448.5 ± 549.5	0/100	0.0	0.0
Jul 8–14	9	6.6 ± 11.6	0/59	9	325.3 ± 131.4	5/800	6.3 (0.8–11.7)	2.0 (0.3–3.8)
Jul 15–21	10	9.0 ± 8.8	0/88	10	1,643.0 ± 899.8	7/1,000	7.0 (1.8–12.2)	11.5 (3.0–20.0)
Jul 22–28	11	7.0 ± 5.9	0/69	8	259.6 ± 301.9	4/1,000	4.0 (0.1–7.9)	1.0 (0.03–2.1)
Jul 29–Aug 4	13	7.1 ± 10.4	0/74	7	169.0 ± 105.3	10/937	10.7 (4.1–17.3)	1.8 (0.7–2.9)

*WNV, West Nile virus: CI, confidence interval.
†Light trap index is mean number of adult female *Cx. tarsalis* mosquitoes collected per trap night. Seven traps were used in 2006, and 5 traps were used in 2007.
‡Infection rate is in units of 1,000 mosquitoes and determined by maximum-likelihood estimate. Infection rate was 0 for all collection dates in 2006.
§Vector index is the product of light trap index and infection rate. Unit of measure is number of infected female *Cx. tarsalis* mosquitoes per trap night. Vector index values were 0 for all collection weeks in 2006.

Table 3. WNV detected by plaque assay or RT-PCR in tissues from American white pelican carcasses collected at Medicine Lake National Wildlife Refuge, Montana, USA, 2006–2007*

Specimen	No. (%) plaque assay positive, n = 27	Median viral titer, log PFU/0.5 cm^3 (range)†	No. (%) RT-PCR positive, n = 8
Feather pulp	18 (66.7)	3.5 (1.3–5.9)	6 (75.0)
Kidney	18 (66.7)	2.6 (2.0–3.9)	3 (37.5)
Spleen	4 (14.8)	1.6 (0.7–2.0)	1 (12.5)
Brain	21 (77.8)	2.7 (1.7–5.9)	5 (62.5)
Heart	14 (51.9)	3.6 (0.7–5.3)	2 (25.0)
Lung	6 (22.2)	2.4 (1.7–3.3)	1 (12.5)
Skin	25 (92.6)	3.1 (0.7–5.0)	5 (62.5)
Oral swab	9 (33.3)	2.1 (0.7–3.7)	3 (37.5)
Cloacal swab	7 (25.9)	1.8 (0.7–3.7)	2 (25.0)
Eye swab‡	2 (11.8)	2.2 (1.7–2.6)	–

*WNV, West Nile virus; RT-PCR, reverse transcription–PCR. All carcasses were WNV positive by virus isolation or detection of specific WNV RNA by RT-PCR.
†Median titers were calculated from the positive specimens only. Unit of measurement for swabs is per swab rather than per 0.5 cm^3.
‡Eye swabs were collected from only 17 WNV-positive chicks.

Most pelican colonies are remote from human population centers and are not currently actively monitored for WNV-associated deaths. Although human population densities near pelican colonies are low, infected host-seeking mosquitoes may travel >10 km in search of a blood meal, especially because breeding pelicans and other birds disperse from the region, typically in August. *Cx. tarsalis* mosquitoes have traveled distances ≤12.6 km (*16*). Other mosquito species, such as *Ae. vexans*, could serve as bridge vectors between infectious juvenile pelicans and susceptible humans. Blood meal analyses from engorged *Ae. vexans* and *Cx. tarsalis* mosquitoes showed that these well-known biters of humans also feed on pelicans. Furthermore, dispersing pelicans may be infectious and introduce the virus to competent mosquitoes near human population centers. Pelicans forage daily ≤80 km from colony sites (*17*).

We confirmed vector-borne transmission of WNV to pelicans at MLNWR in Sheridan County, Montana. *Cx. tarsalis* mosquitoes appeared to be the major vector for transmission of WNV to pelicans in 2007 because vector and trap indices were high for this mosquito species, and blood meal identification linked these vectors to the pelicans. WNV was detected in pelican carcasses in 2006 despite low populations of *Cx. tarsalis* mosquitoes and lack of WNV detection in mosquitoes, which suggested that pelican deaths may be a sensitive indicator of local WNV activity. Juvenile pelican deaths caused by WNV infection have been observed (*4*,*6*) but never targeted for WNV avian mortality surveillance, i.e., to generate public health–related data to be used for WNV prevention and control. In the reports of juvenile pelican deaths, comparison of WNV tissue loads in pelicans was not rigorously evaluated.

Table 4. Virus titers of field-collected samples from WNV-positive American white pelican chicks and test agreement with carcass-matched specimens, Montana, USA, 2006–2007*

Sample	No. (%) WNV positive, n = 19	Median viral titer, log PFU/0.5 cm^3 (range)	κ
Skin†	15 (88.2)	3.6 (1.7–5.0)	0.88
Feather pulp	15 (78.9)	4.9 (1.3–5.6)	0.84
Oral swab	7 (36.8)	1.2 (0.7–2.1)	0.79
Cloacal swab	4 (21.1)	1.5 (0.7–2.7)	0.79

*WNV, West Nile virus; κ, concordance. All carcasses were positive for WNV infection by virus isolation or detection of specific WNV RNA by reverse transcription–PCR. Only plaque assay results are presented for field-collected samples.
†Skin was collected from only 17 carcasses in the field.

Biologic specimens collected from avian carcasses have proven useful in WNV surveillance; the American crow has been a useful sentinel (*2*,*3*,*18*). Oral swabs and feather pulp are preferred target samples for diagnosis of WNV infection in corvids (*19*,*20*). To help guide future WNV surveillance efforts, we sought to determine which pelican samples would be most useful for WNV detection. Our results showed that skin and feather pulp are the most ideal specimens. Previous research showed that feather pulp was slightly more efficacious than oral swabs for detecting WNV when the VecTest assay was used for corvids (*21*), and that 100% of feather pulp samples were positive among WNV-infected American crows and blue jays (*Cyanocitta cristata*) (*20*). Feather pulp and skin meet criteria for a low-resource approach to dead bird surveillance: samples require a minimal amount of time for field collection, dissection of the carcass is not required, exposure of laboratory personnel to infected carcasses is avoided, samples can be easily transported and shipped, and laboratory processing costs can be kept to a minimum. Oropharyngeal, cloacal, and eye swab samples were relatively insensitive for detecting WNV in pelicans.

Continued surveillance of American white pelican colonies is useful for assessing long-term effects of WNV in colonies and in populations in the northern plains and upper Midwest region of the United States. The role of these colonial nesting birds in WNV ecology, and conversely that of WNV on pelican ecology, remains unknown. WNV-amplifying hosts and vectors are generally plentiful at pelican colonies, and recurring chick deaths since 2003 suggest that

WNV-induced reductions in pelican populations will continue. Because juvenile pelicans are likely more susceptible to WNV-associated illness and death than adults, the effects of WNV on pelican population growth would manifest as failed recruitment of new birds into the population in affected colonies, rather than loss of fertile adults. Indirect environmental effects of pelican nest failures are unknown.

The association we observed between WNV disease among pelicans and humans does not imply that pelicans are the source of human WNV infections, or vice versa, and may merely be a consequence of geographic autocorrelation. However, this link highlights the benefit of communication between wildlife and public health sectors. Knowledge of WNV infections in either sector may signal a problem requiring attention in the other. This study of deaths in pelicans caused by WNV serves as a reminder that wildlife disease investigations may play an useful role in mitigating risk for zoonotic infections in humans.

Acknowledgments

We thank J. Rodriguez, T. Rodriguez, S. Cross, E. Madden, and R. Flagan for assistance with bird collections; A. Jackson and J. Dinkins for assisting with necropsies and sample collection; S. Zanto and the Montana Public Health Laboratory for testing mosquitoes; A.W. Layton for access to the necropsy area of the State of Montana Veterinary Diagnostic Laboratory; K. Burkhalter, J. Velez, and G. Young for laboratory support; and B. Biggerstaff and M. Fischer for sharing statistical and epidemiologic expertise, respectively.

This study was supported by the Montana Department of Health and Human Services, the Montana Agricultural Experiment Station, and the Centers for Disease Control and Prevention.

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Murine Typhus in Austin, Texas, USA, 2008

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In August 2008, Texas authorities and the Centers for Disease Control and Prevention investigated reports of increased numbers of febrile rash illnesses in Austin to confirm the causative agent as *Rickettsia typhi*, to assess the outbreak magnitude and illness severity, and to identify potential animal reservoirs and peridomestic factors that may have contributed to disease emergence. Thirty-three human cases of confirmed murine typhus were identified. Illness onset was reported from March to October. No patients died, but 23 (70%) were hospitalized. The case-patients clustered geographically in central Austin; 12 (36%) resided in a single ZIP code area. Specimens from wildlife and domestic animals near case-patient homes were assessed; 18% of cats, 44% of dogs, and 71% of opossums had antibodies reactive to *R. typhi*. No evidence of *R. typhi* was detected in the whole blood, tissue, or arthropod specimens tested. These findings suggest that an *R. typhi* cycle involving opossums and domestic animals may be present in Austin.

Murine typhus, also known as endemic or flea-borne typhus, is caused by *Rickettsia typhi*, a gram-negative, obligate intracellular bacillus. This zoonotic disease is primarily maintained in rodent–flea cycles and is transmitted to humans when infected flea feces contaminate the flea feeding site or other skin abrasions (*1*). After an incubation period of 6–14 days, a nonspecific febrile illness may develop with symptoms of headache, arthralgia, abdominal pain, and confusion. Approximately 50% of patients also report the development of a diffuse macular or maculopapular rash, which starts on the trunk and spreads peripherally (sparing the palms and soles) nearly 1 week after the initial onset of fever and can last from 1 to 4 days. Although the disease is easily treated with doxycycline, it can be severe or even fatal if not diagnosed and treated properly (*2*,*3*).

Throughout its global distribution, *R. typhi* has been primarily concentrated in coastal urban areas where it is maintained among rats (*Rattus* spp.) and oriental rat fleas (*Xenopsylla cheopis*) (*3*). Within the United States, murine typhus is endemic in parts of California, Hawaii, and Texas, where <100 cases are reported annually (*4*–*7*) with a 1%–4% fatality rate when left untreated (*3*,*4*). Recent studies in southern Texas and California indicate that the classic rodent-flea cycle of *R. typhi* has been augmented in these suburban areas by a peridomestic cycle involving free-ranging cats, dogs, opossums, and their fleas (*1*,*6*,*7*). In addition, *R. felis*, which may produce a febrile illness in humans (*8*), may also circulate within these same zoonotic cycles (*7*,*9*). Although both agents have been documented in opossum-flea cycles in parts of southern Texas (*7*,*9*), these diseases are rare in the Austin/Travis County area. Though Austin is only 140 km from the Texas coast, where murine typhus is endemic, only 4 cases have been reported there in the past 25 years; 2 of those 4 cases were reported in September 2007 (Texas Department of State Health Services [TDSHS], unpub. data).

From March through July 2008, the Austin/Travis County Department of Health and Human Services (ATCDHHS) identified 13 cases of febrile illness, half of which had a rash or a severe headache, or both. Laboratory tests conducted at the TDSHS and the Centers for Disease

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DOI: 10.3201/eid1603.091028

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Control and Prevention (CDC) indicated that these patients all had antibodies reactive to *R. typhi*. Active infection with *R. typhi* was also identified in 1 patient by PCR. In August 2008, TDSHS, CDC, and ATCDHHS initiated a detailed epidemiologic investigation to confirm the causative agent as *R. typhi*, to assess the outbreak magnitude and illness severity, and to identify potential animal reservoirs and peridomestic factors that may have contributed to disease emergence.

Methods

In August 2008, TDSHS, CDC, and ATCDHHS initiated an epidemiologic investigation into the emergence of murine typhus in Austin. A clinical investigation was conducted to assess the magnitude and severity of the outbreak. An environmental investigation was conducted to assess the environment and peridomestic factors and domestic animals around case-patient home sites to identify possible means of transmission and risk factors for disease.

Clinical Investigation

Healthcare providers in Austin were asked to report any suspected cases to the health department. Suspected cases were reported to ATCDHHS by the National Electronic Disease Surveillance System. Criteria for suspected cases were high fever (≥38°C), with at least 1 of the following: headache, rash, or myalgia. Confirmed cases were defined as meeting the suspected case criteria and having laboratory confirmation for *R. typhi* infection. The criteria for laboratory confirmation included at least a 4-fold rise in antibody titer to *R. typhi* antigen between paired serum specimens obtained ≥3 weeks apart or the detection of *R. typhi* DNA in a clinical specimen by PCR.

All suspected and confirmed case-patients identified from March through November 2008 were interviewed in-person or by telephone, medical chart reviews were conducted, and serum specimens were collected for laboratory testing. Where the patient was <18 years old, the parents were interviewed. All patients or their proxies were interviewed by using a standard questionnaire. Information collected included demographics, laboratory test results, and clinical symptoms. Medical records of all patients were reviewed. Abstracted data included results of radiographs, urinalyses, blood counts, serologic analysis, and liver enzyme analyses.

Environmental Investigation

Environmental assessments were conducted at the households of 21 case-patients who had been identified from March through July 2008. An external site assessment of the physical property was conducted, including evaluations of environmental factors such as housing structure, vegetation, water features, food sources, and evidence of animals present. When possible, household owners were queried on the internal and external use of pesticides, ownership of domestic animals, use of flea- and tick-control products, history of flea infestations, and reported past evidence of rodents or other types of wildlife in or around the property.

Serum and whole blood specimens were collected from cats and dogs from consenting case-patient households, as well as from feral cats submitted by humane organizations working in the area. A total of 791 trap nights using a combination of live traps (H.B. Sherman Traps, Tallahassee, FL, USA, and Tomahawk Live Trap Co., Tomahawk, WI, USA) were also conducted around 10 case-patient households, targeting capture of peridomestic small wild mammals. In addition, wildlife was accepted from organizations that trapped so-called nuisance species within the outbreak area. Wildlife species were released after specimen collection, except for rats, which were humanely euthanized. Serum and whole blood, as well as ectoparasites, were collected from all animals. Tissue specimens (heart, lung, kidney, spleen and liver) were collected from animals that were euthanized. The address of residence or location was recorded for each animal assessed.

Laboratory Analyses

Confirmatory tests for suspected human cases were performed at a variety of private commercial laboratories; results were then verified by subsequent testing at the TDSHS Laboratory, Austin, Texas, USA, the Rickettsial Zoonoses Branch Diagnostic Laboratory at CDC, Atlanta, Georgia, USA, or both. All animal and arthropod samples were tested at CDC.

Serologic Analysis

Serologic analysis was conducted by using indirect immunofluorecent antibody (IFA) assays for *R. typhi* grown in embryonated chicken yolk sacs, air-dried, and acetone-fixed onto template slide wells. In each assay, antibodies bound to the antigens are detected by using species specific fluorescein isothiocyanate (FITC)–labeled conjugates. We used FITC conjugates (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) produced in goats against human immunoglobulin (Ig) G (γ-chain–specific at a final dilution of 1:150), human IgM (μ-chain–specific at a final dilution of 1:100), rat IgG (heavy plus light [H + L] chain) (diluted at 1:100), mouse IgG (H + L chain) (1:100), cat (H + L chain) IgG (1:100), and a monovalent conjugate against dog IgG (γ-chain–specific) (1:150). FITC-labeled conjugate against opossum IgG (H + L chain) (Bethyl Laboratories, Montgomery, TX, USA) was used at a final dilution of 1:100. The assay format, buffers, and other reagents were used according to the method described by Nicholson et al. (*10*). Samples were serially (*2*) diluted and the last

well demonstrating specific fluorescence of the *R. typhi* organisms was recorded as the endpoint titer (expressed as a reciprocal of the dilution).

Amplification by PCR and Sequencing

Fleas were identified to species, and DNA was isolated from each specimen by using the Biomek 2000 Laboratory Automation workstation (Beckman, Fullerton, CA, USA) and reagents from the Wizard Prep kit (Promega, Madison, WI, USA) (*11*). Detection of *R. felis* and *R. typhi* DNA was conducted by using a TaqMan assay for the citrate synthase (*glt*A) gene of *Rickettsia* spp. as described elsewhere (*11*,*12*). The reactions were conducted by using the Brilliant Q PCR core reagent kit (Stratagene, La Jolla, CA, USA) and run on an *i*Cycler (Bio-Rad, Hercules, CA, USA). Primers and probes were produced by the CDC Core Facility (Atlanta, GA, USA). For animal and human specimens, DNA was extracted from 200 μL of EDTA-blood and 25–50 mg tissue by using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). Animal specimens were tested by *glt*A TaqMan. PCR assays for the rickettsial 17-kDa antigen gene were used for detection of spotted fever and typhus group rickettsiae DNA in clinical specimens with Ready-to-Go-Beads (Amersham Biosciences UK Ltd., Little Chalfont, UK) as described elsewhere (*13*,*14*). Amplicons were purified using Wizard SV Gel and PCR Clean-Up System according to the manufacturer's instructions (Promega). The purified product was sequenced with the ABI PRISM BigDye Terminator Cycle 3.1 Sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequenced product was then purified with a QIAGEN DyeEx 2.0 kit (QIAGEN) and run on an Applied Biosystems 3100x Sequencer (Applied Biosystems).

Results

Clinical Investigation

Thirty-three of 53 patients with suspected cases were confirmed to have murine typhus. All 33 were laboratory confirmed by IFA assay; 1 case was serologically confirmed by PCR, and the sequenced product was positive for *R. typhi* DNA. Illness onset among the patients ranged from March through October 2008, with 70% occurring during May–August (Figure 1). Patients with confirmed cases had an average age of 39 years (range 7–64 years, 15% <18 years); most were male (56%) and white (97%). Although no deaths were attributed to murine typhus among this cohort of case-patients, 23 (70%) were hospitalized (mean 7 days; range 3–14 days), and 9 (27%) were admitted to intensive care units (mean 5 days, range 1–10) with complications, including pneumonia, coagulopathy, and renal failure. Seventeen (51%) patients received antimicrobial drugs, 13 (76%) of them doxycycline. The mean time from symptom onset to antimicrobial drug treatment was 8.3 days (median 8 days, range 1–19 days). No significant differences were detected in rates of hospitalization ($p = 0.78$) and complications ($p = 0.84$) between those patients who did and those who did not receive doxycycline.

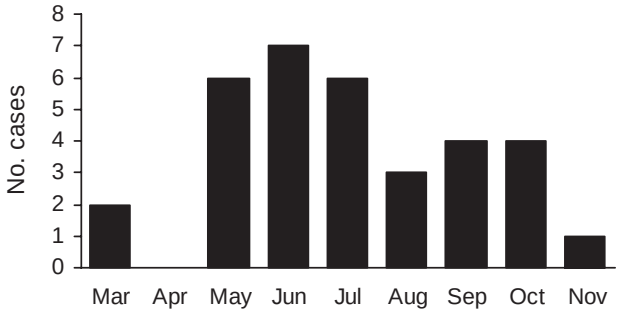

Figure 1. Month of illness onset for laboratory-confirmed murine typhus cases (n = 33) reported in Austin/Travis County, Texas, USA, 2008.

The median high temperature reported among confirmed case-patients was 40°C (range 39°C–41°C). The most commonly reported symptoms included malaise (76%), headache (73%), chills (61%), and myalgia (61%). Loss of appetite (58%), nausea (52%), rash (46%), vomiting (42%), and diarrhea (36%) were also reported by many case-patients. Less than one third of case-patients reported photophobia (30%), arthralgia (33%), stiff neck (24%), backache (21%), abdominal pain (21%), coughing (18%), jaundice (18%), lymphadenopathy (15%), conjunctivitis (12%), and confusion (12%). Serologic results showed that impaired liver function was common in patients (70%), and some had impaired kidney function (21%).

The 33 confirmed case-patients clustered geographically in central Austin (Figure 2). Twelve (36%) resided in 1 ZIP code area in a suburban-residential area (Table 1). Most other patients were from adjacent and nearby central and east central Austin areas. One case-patient resided in northern Travis County but worked in central Austin.

Environmental Investigation

Twenty-six (79%) of the 33 confirmed case-patients owned a dog or cat. Of those, 14 (42.4%) reported regularly administering flea/tick preventatives to their pets. However, only 2 patients (5.4%) noted flea bites or exposure in the 2-week period before illness onset. Recent exposure to opossums was reported by 11 (29.7%) of the patients; >20% had been recently exposed to rats, 19% to raccoons, and 5% to mice through both direct and indirect contact.

External site assessments were performed at 20 home sites (representing 21 case-patients). Of the home sites evaluated, 9 (45%) had pet food outside; 9 (45%) had a garden

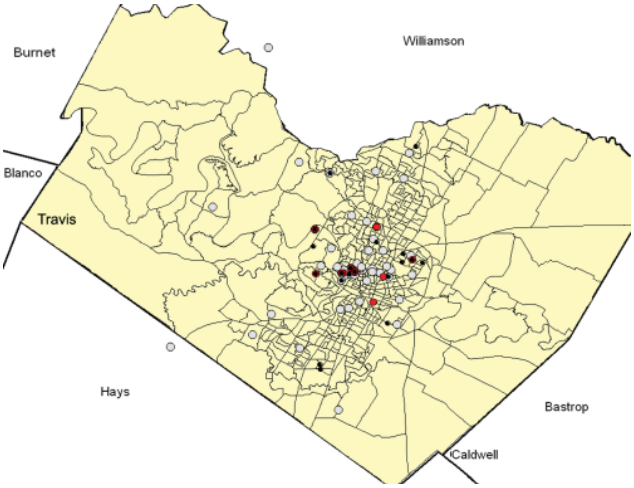

Figure 2. Distribution of confirmed murine typhus case-patients and animals by serologic status for antibodies to *Rickettsia typhi* in Travis County, Texas, USA, 2008. Red circles, confirmed case-patients; black circles, seropositive animals; gray circles, seronegative animals.

or compost heap; 12 (60%) had outdoor piles of firewood or harborage; 12 (60%) had apparent evidence of rodents (through direct observations or the presence of feces, nests, or burrows); 17 (85%) had outdoor water sources; and 17 had unsecured garbage outside.

A total of 56 animals (including 17 cats, 9 dogs, 17 opossums, 9 raccoons, and 4 rats) (Table 2) and 139 arthropods were obtained; all but 1 opossum was evaluated (Table 3). Overall, 19 (33.9%) of all animals tested were seropositive. This sample included 3 (17.6%) cats, 4 (44.4%) dogs, and 12 (70.6%) oppossums. None of the samples obtained from raccoons or rats were seropositive. Seropositive animals came from 5 ZIP code areas, and 68% of all seropositive animals came from 2 ZIP codes areas where 35% of the human cases were reported (Figure 2; Table 1). All 3 seropositive cats came from 1 capture site, whereas the 4 seropositive dogs were owned by 3 case-patients (2 dogs by a single patient) from 3 ZIP code areas. Seropositive opossums were from 8 capture sites in 3 ZIP code areas. Of the arthropods evaluated, 83.5% were identified as *Ctenocephalides felis*, the cat flea (Table 3). No evidence of either *R. typhi* or *R. felis* DNA was detected in any of the whole blood, tissue, or arthropod specimens tested.

Discussion

Murine typhus is a common zoonotic disease in endemic foci of southern Texas, where a mean of 48 cases were reported annually from 1990 through 2006 (*15*). However, before this investigation, murine typhus was not believed to occur commonly in the Austin/Travis County area, and only 2 cases were identified before 2007. This investigation identified 33 patients with laboratory-confirmed cases, nearly 70% of whom were hospitalized from March through November 2008. In addition, 2 murine typhus cases reported in Austin in September 2007 likely represent some of the first cases associated with this emergent focus. These findings represents the first large-scale outbreak reported in Austin/Travis County since eradication efforts were coordinated in this part of Texas in the 1940s (TDSHS, unpub. data).

The clinical features and age distribution of case-patients reported here are similar to those found in case-

Table 1. Distribution by ZIP code of confirmed human murine typhus case-patients and animals and ectoporasites that were tested for *Rickettsia typhi* by IFA assay and/or PCR, Austin/Travis County, Texas, USA, August 2008*

ZIP code	No. human case-patients, n = 33	Opossum (12/17)†	Raccoon (0/9)†	Rat (0/4)†	Cat (3/17)†	Dog (4/9)†	Flea (0/139)†
78702	1	1/1	–	–	–	–	0/14
78703	12	6/7	0/5	0/4	0/2	0/3	0/58
78704	1	–	–	–	–	–	–
78705	5	–	–	–	–	–	–
78722	2	–	–	–	3/4	0/1	0/6
78723	1	0/2	–	–	0/3	1/2	0/15
78727	1	–	–	–	–	–	–
78728	1	–	–	–	0/2	–	0/5
78731	1	–	–	–	–	–	–
78741	0	–	0/1	–	–	–	0/2
78745	0	–	0/1	–	–	–	0/4
78746	2	5/7	0/2	–	0/2	2/2	0/31
78747	1	–	–	–	–	–	–
78748	0	–	–	–	0/1	–	0/2
78751	2	–	–	–	–	–	–
78757	3	–	–	–	0/1	1/1	0/2
78759	0	–	–	–	0/2	–	–

*IFA, immunofluorescent antibody.
†Values are no. positive/no. tested except as indicated.

Table 2. Frequency and distribution of animals seropositive for *Rickettsia typhi*, Austin/Travis County, August, 2008*

Animal	No. animals tested	No. (%) animals seropositive	IFA assay titer, no. animals								
			<32	32	64	128	256	512	1,024	2,048	≥4,096
Cat	17	3 (17.7)	14	0	0	0	0	1	0	0	2
Dog	9	4 (44.4)	5	0	0	0	0	1	2	1	0
Opossum	17	12 (70.6)	5	4	3	1	3	1	0	0	0
Raccoon	9	0	9	0	0	0	0	0	0	0	0
Rat	4	0	4	0	0	0	0	0	0	0	0
Total	56	19 (33.9)	38	4	3	1	3	3	2	1	2

*IFA, immunofluorescent antibody. Seropositivity indicated by titer ≥32.

patients reported in other murine typhus studies (*4*,*16*). Although 70% of the case-patients identified during this outbreak were hospitalized, this percentage is slightly less than what was observed by Taylor et al. (*16*) during a study of 200 cases in Texas from 1980 through 1984, in which 85% of patients were hospitalized and 1% died. Though no deaths were reported during this 2008 outbreak, nearly one third of all patients were admitted to the intensive care unit with complications (including pneumonia, coagulopathy, and renal failure) that demonstrated the severity of illness.

Delaying treatment for murine typhus increases the duration of symptoms and risk for complications (*4*,*17*). Treatment should always be initiated on the basis of clinical and epidemiologic considerations alone without waiting for a laboratory confirmation of the diagnosis. In this outbreak, 48% of patients did not receive treatment with doxycycline, the drug of choice for treatment for rickettsial diseases. The lack of doxycycline administration and the reported lag time of 1 week to nearly 3 weeks between symptom onset and antimicrobial drug treatment experienced by most patients may have been associated with a delay in recognizing that the cases were murine typhus, because of the perception that the disease was not present in Austin. Despite this finding, the difference in hospitalization and complication rates did not appear to be significant between patients with and without proper antimicrobial drug treatment. However, the small sample size may have precluded a robust comparison of these data.

Strong serologic evidence of exposure to rickettsiae was detected among opossum and domestic animal populations in Austin/Travis County. More than one third of all animals tested were seropositive with *R. typhi* antigen. Of particular interest, >70% of opossums tested were seropositive with *R. typhi* antigen. Further studies are needed to determine the specific role that opossums play in the ecology of murine typhus in the Austin area. Exposure to other rickettsiae in the spotted fever group also cannot be excluded, particularly for *R. felis*, which is very common in cat fleas obtained from opossums (*7*,*12*). The serologic findings observed here are similar to what has been observed in studies of disease-endemic regions in southern Texas and California, USA, where opossums are hosts for fleas containing *R. typhi* and *R. felis* (*6*,*7*,*9*,*18*). In Los Angeles, California, and Corpus Christi, Texas, 42% and 25% of opossums were found to be seropositive for *R. typhi*, respectively, although seropositive rats were rarely or never detected (*7*,*9*). These studies have resulted in a reevaluation of the classic urban cycle of murine typhus in suburban disease-endemic areas in the continental United States, where opossums, domestic cats, and cat fleas—and not rodents and their fleas—are considered to be a primary source of infection (*2*).

Although none of the rats in this study were seropositive for *R. typhi*, the small sample size tested (n = 4) limits our ability to draw conclusions regarding the contribution of rats and their arthropods to the dynamics of murine typhus in this area. Additionally, presumptions regarding contributions of various animal species are limited because only serologic findings were positive; active infection with either *R. typhi* or *R. felis* was not detected in any of the samples tested. While none of the fleas were positive for either *R. typhi* or *R. felis* DNA, this result is not entirely unexpected considering the infrequency with which positive

Table 3. Summary of fleas collected from animals in Austin/Travis County, Texas, USA, August 2008

Animal	No. animals with fleas/total no. animals	Flea species collected	Total no. fleas collected	Frequency of flea species by host animal, %	Infestation index
Cat	12/17	*Ctenocephalides felis*	23	70.6 (n = 12)	1.35
Dog	1/9	*Ctenocephalides canis*	1	11.1 (n = 1)	0.11
Opossum	18/18	*C. felis*	84	100.0 (n = 18)	4.67
		Pulex irritans	14	22.2 (n = 4)	0.78
Raccoon	9/9	*C. felis*	8	44.4 (n = 4)	0.89
		Echidnophaga gallinacean	1	11.1 (n = 1)	0.11
		P. irritans	5	33.3 (n = 3)	0.56
		Xenopsylla cheopis	2	22.2 (n = 2)	0.22
Rat	1/4	*C. felis*	1	25.0 (n = 1)	0.25
Total	41/56	–	139	73.2 (n = 44)	–

fleas were detected in similar studies. For instance, Boostrom et al. (*7*) identified only 3 *R. typhi* and 11 *R. felis* positive fleas out of a sample of 529 from highly endemic parts of southern Texas. Still, *R. felis* may be circulating within this area because both pathogens appear to be maintained in complex ecologic cycles (*2*,*7*). More specific studies targeting larger numbers of statistically representative domestic animals and wildlife are needed to better discern complicated human-animal-disease dynamics.

Murine typhus may now be established in the Austin/Travis County area and should be considered an ongoing public health threat. Although, the idea that persons have been infected with *R. felis* (which has been previously found to infect a patient in Texas) cannot be totally excluded (*8*). Continued public health education efforts are needed in the Austin/Travis County area regarding the emergence of flea-borne rickettsiosis and the likely risk factors for infection, with an emphasis on avoiding contact with wild animals and controlling fleas on pets and around the home with approved products. Physicians in the area should maintain an increased vigilance in detecting and diagnosing suspected murine typhus cases as well as other rickettsioses, because timely treatment with the appropriate antimicrobial drug therapy is critical for limiting severe outcomes.

Acknowledgments

We thank the following persons and organizations for their support throughout this investigation: Eda Gowdy, Philip Huang, Joe Staudt, Melissa Maass, Guy Moore, Beverlee Nix, Tom Sidwa, Glenna Teltow, Maria L. Zambrano, Joseph Singleton, Sandor E. Karpathy, John Krebs, Gregory A. Dasch, Robert Massung, and staff from the Austin Humane Society and the Town Lake Animal Shelter, Austin, Texas.

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Chikungunya Virus Infection during Pregnancy, Réunion, France, 2006

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Mother-to-child transmission of chikungunya virus was reported during the 2005–2006 outbreak on Réunion Island, France. To determine the effects of this virus on pregnancy outcomes, we conducted a study of pregnant women in Réunion in 2006. The study population was composed of 1,400 pregnant women (628 uninfected, 658 infected during pregnancy, 27 infected before pregnancy, and 87 infected on unknown dates). We compared pregnancy outcomes for 655 (628 + 27) women not infected during pregnancy with 658 who were infected during pregnancy. Infection occurred during the first trimester for 15% of the infected women, the second for 59%, and the third for 26%. Only hospital admission during pregnancy differed between infected and uninfected women (40% vs. 29%). Other outcomes (cesarean deliveries, obstetric hemorrhaging, preterm births, stillbirths after 22 weeks, birthweight, congenital malformations, and newborn admissions) were similar. This virus had no observable effect on pregnancy outcomes.

Chikungunya virus infection is transmitted by mosquitoes of the genus *Aedes*. The virus was first isolated in 1952 and is found in eastern Africa, India, and Southeast Asia. Symptoms of infection are high fever and disabling muscle and joint pain, often associated with a rash and mild bleeding. Persons infected usually recover spontaneously in several days to a week (*1*). Fever and arthralgia may occur for several months or even years (*2*). Patients are treated only for their symptoms because there is no specific treatment for the underlying infection (*3*). Before the recent outbreak on the island of Réunion, the disease was not considered life-threatening.

Réunion, a French territory in the southwestern Indian Ocean, has a population of ≈785,000 inhabitants. Medical facilities in Réunion are similar to those in mainland France and other industrialized countries. A major chikungunya outbreak occurred in Réunion in 2005–2006. At the end of this outbreak, seroprevalence was estimated to be 38.2% (95% confidence interval [CI] 35.9%–40.6%); 300,000 (95% CI 283,000–320,000) persons were infected (*4*,*5*). *Aedes albopictus* mosquitoes were the primary vector in this outbreak.

The outbreak began in eastern Africa (*6*). It reached Réunion in March 2005 but was relatively inactive, with only several thousand cases until November 2005, when its incidence unexpectedly increased during summer in the Southern Hemisphere, peaking at 47,000 cases/week during week 5 of 2006. The most recent cases were reported in August 2006. Comparisons of 2006 with previous years showed that mortality rates increased during February, March, and April 2006 (*7*,*8*). Since 2006, the virus has caused several epidemics in the Indian Ocean region (Madagascar, India, Sri Lanka, Thailand, Malaysia, and Singapore). Three new cases of chikungunya were reported in August 2009 on Réunion Island (*9*).

The first cases of virus transmission from mother to child at birth were identified in February 2006; a total of 38 such cases were reported (*10*,*11*). The virus was also found

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DOI: 10.3201/eid1603.091403

in specimens from 3 early second trimester miscarriages (*12*). When this outbreak began, little information was available about the risk for chikungunya virus infection in pregnant women. In addition to virus transmission at birth, potential complications include transplacental transmission before birth, congenital malformations, stillbirths, growth restriction, and preterm delivery. Chikungunya virus belongs to the same family of viruses (*Togaviridae*) as rubella virus, for which some of these complications have been described (*13*). The high fever that characterizes chikungunya infection could cause uterine contractions or fetal heart rate abnormalities, which might promote spontaneous or induced preterm delivery (cesarean for fetal salvage). The hemorrhagic syndrome described at the onset of infection might be manifested by vaginal bleeding during pregnancy or third-stage hemorrhaging, as reported for infection with dengue virus (*14*,*15*). The proportion of symptomatic and asymptomatic infections was also unknown.

The purpose of our study (the Chikungunya-Mère-Enfant cohort study) was to determine the consequences of chikungunya infection on pregnancy outcomes. These results will be useful to public health officials and physicians who provide care for pregnant women or newborns because chikungunya can be imported by international travelers and the location of *Ae*. *albopictus* mosquitoes has extended beyond the tropics (*16*). These mosquitoes are found in 26 states in the United States and several countries in Europe, where outbreaks are possible (*17*,*18*).

Methods

We began our study in early April 2006, by planning to recruit all pregnant women (with or without symptoms of chikungunya infection) who received care at 1 of the 6 main maternity units in Réunion. These 6 units accounted for 78% of 14,077 live births in Réunion in 2006. Inclusion in the study was proposed regardless of the reason for a visit or admission. We had planned to include 3,600 women so that sufficient children with in utero chikungunya infection were available to study their psychomotor development. To show a difference of 10 points in the developmental quotient at 24 months of age, it would have been necessary to observe 19 children infected in utero. However, because of the decrease in the outbreak after June 1, we revised our sample size and included only pregnant women who reported clinical signs suggestive of this infection. The study cohort was composed of 1,400 pregnant women (mean term 32 weeks); 1,384 (99%) gave birth in 1 of the 6 participating maternity units. Information on pregnancy outcome for 16 women lost to follow-up was obtained by contacting each one directly. A total of 914 participants were included in April, 386 in May, 88 in June, 5 in July, 2 in August, 4 in September, and 1 in November. In an ancillary study, for 3 days in May 2006, all women who gave birth in the 6 participating units were interviewed to determine how women in the study cohort differed from those not in the study in terms of chikungunya symptoms, parity, age, gestational age of the infant at birth, and mode of delivery.

Serologic status for chikungunya virus infection was determined at participant's inclusion in the study. All reports of chikungunya fever were confirmed by using serologic testing or detection of the viral genome in any specimen by using real-time reverse transcription–PCR (RT-PCR) (*19*,*20*). Serologic tests with negative results at inclusion were repeated at delivery or when symptoms suggestive of infection appeared. Histologic examinations were performed on placentas of all women who had chikungunya infection during pregnancy. RT-PCR was also performed for placenta and amniotic fluid samples from women with symptoms at delivery.

Date of infection was determined by checking patient history of symptoms or by RT-PCR when available. Women were classified into 2 groups: those infected by chikungunya virus during pregnancy (symptoms during pregnancy confirmed by positive serologic or RT-PCR results) and those not infected (negative serologic results at delivery or during the preceding 7 days). Women infected before pregnancy were considered not infected during pregnancy. We excluded women who were infected but asymptomatic, those whose symptoms could not be dated, and those with inconclusive serologic results from analysis.

We analyzed how women infected by chikungunya virus during pregnancy (658) differed from those who were not infected (655) for general characteristics (age, educational level, marital status, and body mass index), medical history (diabetes and hypertension), and obstetric history (previous pregnancies, history of preterm delivery, small-for-gestational-age, or stillbirths). We then compared pregnancy outcomes (prenatal hospital admission for any reason and for chikungunya symptoms, vaginal bleeding during pregnancy, mode of delivery, obstetric hemorrhage, stillbirth, preterm birth, birthweight, congenital malformations, and newborn hospitalization) between the 2 groups. Obstetric hemorrhage was defined as blood loss >500 mL. We considered only fetal malformations recognized by European Surveillance of Congenital Abnormalities (EUROCAT) (www.eurocat.ulster.antibodies.uk). All malformations recorded were verified by checking either pediatric files or the Réunion congenital anomalies registry, which is affiliated with EUROCAT.

Bivariate analysis of pregnancy outcomes compared means (by Wilcoxon rank-sum test) and percentages (χ^2 or Fisher exact tests). For multivariate analysis, we adjusted for center, maternal age, educational level, and body mass index. Logistic regression was used to estimate the adjusted odds ratios (ORs). A p value <0.05 was considered significant. Sensitivity analyses were performed to deter-

mine whether results changed when either the 27 infected before pregnancy or the 100 women included in the study after May 2006 were omitted from the analysis. Statistical analysis was performed by using SAS version 9.1 software (SAS Inc., Cary, NC, USA).

This prospective multicenter study was reviewed and approved by the ethics committee (Comité de Protection des Personnes) of Tours (no. 2006–2007). It was reported to the French Data Protection Authority (Commission Nationale de l'Informatique et des Libertés).

Results

Of 1,400 pregnant women included in the study, 705 (50%) reported chikungunya symptoms during pregnancy, 668 (48%) reported no symptoms, and 27 (2%) reported symptoms before pregnancy (Table 1). Specific serologic or RT-PCR tests confirmed the diagnosis of chikungunya infection for 658 (93%) of 705 who reported symptoms during pregnancy. In 6 cases (1%), serologic results for immunoglobulin (Ig) G were negative at delivery, which ruled out infection. Conclusions could not be reached for 41 women (6%) because of missing or inconclusive laboratory data. Negative serologic findings for IgG confirmed the absence of chikungunya infection in 622 (93%) of 668 women with no reported symptoms during pregnancy. Findings were positive for 46 women (7%); these women were considered asymptomatically infected at an unknown date and excluded from the analysis. Chikungunya infection was confirmed for all 27 women with symptoms before pregnancy. Overall, 658 women were classified as infected by chikungunya virus during pregnancy (exposed) and 655 as not infected during pregnancy (not exposed).

Among the 658 exposed women, infection occurred during the first trimester for 99 (15%) women, the second for 387 (59%), and the third for 172 (26%). Infection occurred during the first quarter of 2006 for 536 (81%), before that for 62 (9.4%), and after that for 60 (9.1%). Maternal signs and symptoms were fever (408 cases, 62%), arthralgia (615 cases, 93%), headache (354 cases, 54%), edema (355 cases, 54%), diarrhea (78 cases, 12%), aphthae (63 cases, 9.6%), epistaxis or gingivorrhagia (59 cases, 9.0%), and rash (496 cases, 76%). Overall, 137 (21%) were hospitalized for chikungunya infection for a median duration of 2 days (range 1–75 days). Signs of infection began a median of 108 days before delivery (range 1–263 days), and only 4 infected women (0.6%) had symptoms in the 7 days before delivery.

Pregnancy outcomes included 656 live births to women who were infected and 653 to those who were not infected (including 8 and 14 pairs of twins, respectively); 5 and 8, respectively, stillbirths after 22 weeks of gestation, and 5 and 8, respectively, miscarriages before 22 weeks. Of the 4 children born to mothers infected by chikungunya during the last week of pregnancy, 1 newborn had signs of infection on the third day of life, and RT-PCR and IgM serologic analysis confirmed the infection. The mother had had chikungunya symptoms the day before delivery. The other 3 neonates remained asymptomatic and had no detectable IgM against chikungunya virus. Of 624 placentas examined from women found to be infected during pregnancy, only the placenta from the case of mother-to-child transmission had histologic signs compatible with viral infection.

Table 1. Chikungunya virus infections in 1,400 pregnant women, by onset or lack of symptoms, Réunion, France, 2006*

Symptoms	No. infected	Diagnosis
Symptoms during pregnancy, n = 705		
Yes	658	Exposed
No	6	Not exposed
Unknown	41	Excluded
No symptoms, n = 668		
Yes	46	Excluded
No	622	Not exposed
Symptoms before pregnancy, n = 27		
Yes	27	Not exposed

*Infection was confirmed by positive serologic or reverse transcription–PCR results. Women infected before pregnancy were considered not infected during pregnancy.

RT-PCR was performed to test for the viral genome in the placenta or amniotic fluid from 3 of the 5 stillbirth fetuses (>22 weeks) of women with chikungunya infections. The test result was positive in 2 cases, in which chikungunya symptoms in the mothers had begun 25 and 70 days before the fetal loss. For the 8 miscarriages before 22 weeks, RT-PCR was performed on trophoblast tissue for 1 case and the result was negative.

Women infected by chikungunya during pregnancy were more likely to have been born in Réunion, to have stopped going to school at a younger age, to be unmarried, overweight, or already have children (Table 2). They also differed by maternity center. Multivariate analysis showed that only 2 characteristics were significantly different: educational level (primary school OR 1.48, 95% CI 1.11–1.97; high school as reference; university OR 0.54, 95% CI 0.38–0.77) and being overweight (body mass index $\geq$25 kg/m^2, OR 1.76, 95% CI 1.22–2.55).

After we controlled for potential confounders, the only difference in pregnancy characteristics between infected and uninfected women (Table 3) was the frequency of hospital admissions during pregnancy (40% vs. 29%). This difference disappeared when hospital admission for suspected chikungunya was excluded (26% vs. 28%). Other maternal and neonatal outcomes were similar in both groups. Excluding women infected before pregnancy or included after May 2006 from the analysis did not modify the results (Table 3). Congenital malformations observed in newborns as a function of maternal exposure are shown in Table 4.

In early May, we conducted a 3-day survey of all women giving birth in the maternity units participating in the study. Of 113 women interviewed, 43% (49) were included in the study cohort. The inclusion rate differed according to maternity unit, ranging from 16% to 88%. The mean proportion of women asked to participate was 62% (70/113),

Table 2. Characteristics of women infected and not infected with chikungunya virus during pregnancy, Réunion, France, 2006*

Characteristic	Infected, no. (%), n = 658	Not infected, no. (%), n = 655	p value†
Born in Réunion			
Yes	545 (84.1)	510 (79.2)	
No	103 (15.9)	134 (20.8)	0.02
Education			
Primary school	331 (52.2)	214 (34.3	<0.0001
High school	198 (31.2)	200 (32.1)	
University	105 (16.6)	209 (33.6)	
Marital status			
Lives alone	252 (39.0)	207 (32.0)	0.008
Lives with partner	394 (61.0)	440 (68.0)	
History of diabetes			
Yes	17 (2.6)	14 (2.1)	
No	641 (97.4)	641 (97.9)	0.59
History of hypertension			
Yes	23 (3.5)	27 (4.1)	
No	635 (96.5)	627 (95.9)	0.55
Previous pregnancies <22 wks			
Yes (≥1)	273 (41.6)	258 (39.5)	
No	384 (58.4)	395 (60.5)	0.45
Mean parity	1.4 (1.6)	1.1 (1.4)	<0.0001
0	216 (32.9)	278 (42.7)	0.0004
1	199 (30.3)	181 (27.8)	
2	110 (16.8)	106 (16.3)	
≥3	131 (20.0)	86 (3.2)	
Previous stillbirth or neonatal death			
Yes	22 (3.3)	12 (1.8)	
No	636 (96.7)	643 (98.2)	0.08
Previous preterm delivery			
Yes	44 (6.7)	27 (4.1)	
No	614 (93.3)	626 (95.9)	0.04
Previous child >2,500 g			
Yes	70 (10.7)	55 (8.4)	
No	587 (89.3)	598 (91.6)	0.17
Previous cesarean			
Yes	71 (10.8)	66 (10.1)	
No	587 (89.2)	586 (89.9)	0.69
Mean age at delivery, y	28.6 (6.9)	28.8 (6.4)	0.52
<20	71 (10.8)	69 (10.5)	0.94
20–29	309 (47.0)	303 (46.3)	
≥30	278 (42.2)	283 (43.2)	
Mean body mass index, kg/m^2	24.7 (5.9)	23.4 (5.1)	<0.0001
<25	390 (60.8)	454 (71.5)	<0.0001
25–29	137 (21.3)	113 (17.8)	
≥30	115 (17.9)	68 (10.7)	
Center			
1	165 (25.1)	188 (28.7)	<0.0001
2	196 (29.8)	153 (23.4)	
3	62 (9.4)	71 (10.8)	
4	21 (3.2)	9 (1.4)	
5	118 (17.9)	182 (27.8)	
6	96 (14.6)	52 (7.9)	

*Women infected before pregnancy were considered not infected during pregnancy.
†By Wilcoxon rank-sum test for continuous variables and χ^2 test for nominal variables.

Table 3. Pregnancy outcome according to chikungunya virus infection during pregnancy, Réunion, France, 2006*

Characteristic	Infected,† no. (%), n = 658	Not infected,‡ no. (%), n = 655	p value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Hospital admission during pregnancy					
Yes	266 (40.4)	191 (29.2)		1.65 (1.31–2.07)	1.52 (1.18–1.95)
No	392 (59.6)	464 (70.8)	<0.0001	1	1
Hospital admission during pregnancy, suspected infection with chikungunya virus excluded					
Yes	180 (28.0)	136 (26.1)		0.91 (0.70–1.18)	0.83 (0.62–1.10)
No	464 (72.0)	385 (73.9)	0.48	1	1
Vaginal bleeding during pregnancy					
Yes	55 (8.4)	68 (10.4)		0.79 (0.55–1.15)	0.94 (0.63–1.42)
No	596 (91.6)	584 (89.6)	0.22	1	1
Obstetric hemorrhaging					
Yes	36 (5.6)	42 (6.5)		0.85 (0.54–1.35)	0.87 (0.53–1.42)
No	609 (94.4)	605 (93.5)	0.49	1	1
Mode of delivery§					
Vaginal	545 (83.8)	530 (81.5)	0.27	1	1
Cesarean	105 (16.2)	120 (18.5)		0.85 (0.64–1.14)	0.77 (0.56–1.06)
Mean gestational age, wk§	39.0 (2.1)	38.9 (2.5)	0.55		
<32	8 (1.2)	15 (2.3)	0.26	0.52 (0.22-1.24)	0.48 (0.19–1.23)
32–36	53 (8.2)	60 (9.2)		0.86 (0.59–1.27)	0.78 (0.51–1.20)
≥37	589 (90.6)	575 (88.5)		1	1
Mean birthweight, g§	3,116 (549)	3.056 (620)	0.27		
<2,000	20 (3.1)	32 (4.9)	0.36	0.62 (0.35–1.11)	0.66 (0.36–1.22)
2,000–2,999	235 (35.9)	236 (35.7)		0.99 (0.79–1.25)	1.01 (0.79–1.30)
3,000–3,999	372 (56.9)	371 (56.1)		1	1
≥4,000	27 (4.1)	22 (3.3)		1.22 (0.69–2.19)	1.25 (0.65–2.39)
Stillbirth after 22 wk§					
Yes	5 (0.8)	8 (1.2)		0.63 (0.20–1.93)	0.61 (0.18–2.07)
No	653 (99.2)	656 (98.8)	0.41	1	1
Congenital malformation					
Yes	19 (2.9)	15 (2.2)		1.36 (0.68–2.74)	1.54 (0.68–3.49)
No	647 (97.1)	654 (97.8)	0.48	1	1
Admission to neonatal care§					
Yes	53 (8.1)	55 (8.3)		0.97 (0.65–1.44)	1.03 (0.67–1.58)
No	605 (91.9)	609 (91.7)	0.88	1	1

*OR, odds ratio; CI, confidence interval. OR was adjusted for center, educational level, body mass index, and maternal age. Women infected before pregnancy were considered not infected during pregnancy.
†Of the 658 women who were infected, 650 had delivered a child after 22 weeks; 658 children were delivered by these women.
‡Of the 655 women who were not infected, 650 had delivered a child after 22 weeks; 664 children were delivered by these women.
§Miscarriage before 22 weeks was excluded.

and the mean acceptance rate was 70% (49/70); 43% (21) of the women included thought that they had had chikungunya infection during pregnancy compared with 6% (4) of those not included (p<0.0001). Mean parity (2.1 vs. 2.6; p = 0.08), mean maternal age (28.6 years vs. 29.1 years; p = 0.70), mean gestational age at delivery (39.1 weeks vs. 38.7 weeks; p = 0.14), and mode of delivery (18% vaginal vs. 17% cesarean; p = 0.87) did not differ between the women who were or were not included.

Discussion

In this comparative study, we did not observe any effect of chikungynya infection on pregnancy outcomes except for the number of prenatal maternal hospital admissions for chikungunya symptoms. Our study involved a high proportion of maternity units and births in Réunion. Women included in the study in April 2006 accounted for 73% (905/1,240) of all live births in Réunion. Systematic determination of serologic status by identification of specific IgM and IgG confirmed infection status. All patients for whom chikungunya infection during pregnancy was uncertain were excluded. We excluded women who had positive serologic results but did not report symptoms or have a positive RT-PCR result because we could not identify the date of infection. Studies during the outbreak in Réunion showed that IgM tended to persist for 12 to 24 months and cannot be used to identify the date of infection (*21*).

Because inclusion in the study began in April 2006 after the outbreak had peaked, we could not analyze pregnancies completed before this date. Therefore, our study does not describe the consequences of the outbreak on the risk for miscarriage or preterm delivery during the first quarter of 2006. The study included only pregnancies with outcomes after that quarter. Most of the women were infected before

Table 4. Congenital malformation classification, according to ICD-10 code, as a function of maternal exposure to chikungunya virus during pregnancy, Réunion, France, 2006*

Classification	Exposure to chikungunya virus during pregnancy, no. newborns, n = 34	
	Yes	No
Chromosomal (Q90, Q91, Q96)	3	1
Neural tube (Q03, Q05)	3	0
Cardiovascular (Q20, Q21, Q25, Q26)	5	1
Kidneys, urinary tract, genital organs (Q53, Q55, Q61, Q62, Q63)	1	5
Limbs, thorax, bones, and spine (Q66, Q69, Q71, Q74, Q76)	5	9
Ear, cleft palate (Q17, Q35)	3	0
Other (D22, Q33, Q40, Q42, Q89, T21)	4	3

*ICD-10, International Classification of Diseases, 10th revision. Total exceeds 34 because 1 child had 3 types of malformations and 7 children had 2 types.

their inclusion. The fact that many women seen in May had already been included at a previous visit in April explains why there were fewer inclusions in May; only pregnant women seen for the first time or who for some reason had not been included in April were eligible. A disadvantage of conducting a study during an outbreak is that its duration cannot be known in advance. For this reason, the number of women was smaller than planned.

Date of infection was estimated by recording the time of symptoms and confirmed by RT-PCR and serologic testing. The positive predictive value of symptoms was reliable because infection was confirmed in ≈93% of women with suggestive symptoms and ruled out in <1% of these women. The negative predictive value was also reliable because serologic results were negative for 93% of the women without symptoms. These values are similar to the positive predictive value (91%) and negative predictive value (87%) of symptoms observed in a survey of a representative sample of the population in Réunion at the end of 2006 (*4*). These results confirm that clinical signs of chikungunya have an excellent predictive value during an outbreak.

Women who thought that they had had chikungunya infection during their pregnancy because they had symptoms were more likely to agree to participate in the cohort than the women without such symptoms. There were also disparities in the inclusion rate according to maternity center. Because of these differences, women included in this study were not representative of the population of pregnant women during this period in Réunion. These differences in the inclusion rate according to symptoms and hospital make it impossible to estimate the attack rate of infection among the population of pregnant women. However, because other characteristics (parity, age, gestational age at delivery, mode of delivery) were similar, sampling did not create any bias for comparisons between exposed and unexposed women.

The rarity of placental histologic lesions (in only 1 of 624 women with chikungunya infection during pregnancy) confirmed the absence of placental infection by the virus and explained the rarity of cases of fetal chikungunya infection before birth (*22*). Couderc et al. recently showed that human syncytiotrophoblast tissue is refractory to chikungunya infection (*23*). During the outbreak in Réunion, only 3 cases of fetal chikungunya infection at the beginning of the second trimester were reported (*12*). All other reported cases involved symptomatic newborns with chikungunya infection in the days after birth, for whom the presumed mechanism of viral transmission was direct passage from maternal blood into the fetal circulation through placental breaches during labor (*11*). Kwiek and others showed that maternal–fetal microtransfusions that occur during labor promote HIV-1 transmission from mother to child (*24*).

Our results are consistent with those of Gérardin et al., who showed that most cases of maternal–fetal transmission of chikungunya virus occurred at birth (*22*). Because we systematically determined chikungunya serologic status, we could compare pregnancy outcomes between infected and uninfected women. We found no difference in risk for hospitalization (except for suspected chikungunya), preterm delivery, low birthweight, or admission to neonatal care. However, the number of women tested enabled us to show a difference of 7% for prevalence of admission during pregnancy, 5% for preterm delivery, 82 g for fetal weight, and 5% for admission to neonatal care ($\beta = 0.20$ and $\alpha = 0.05$).

Stillbirths were not more frequent among women with chikungunya infection during pregnancy than among uninfected women, even though >62% of infected women had fevers. This observation appears to conflict with the hypothesis that fever plays a direct role in in utero deaths. However, because of the rarity of this event (0.64% in 2002 in Réunion) (*25*), the power of the study is insufficient to justify any definitive conclusion.

In our sample, the minimum detectable difference was 1.8% for stillbirths (0.6% vs. 2.4%, $\beta = 0.20$ and $\alpha = 0.05$). For early fetal loss before 22 weeks, the number of events (13/1,313 women) was lower than the number expected probably because most participants were included after that term. For this reason, we could not analyze outcome and reach a conclusion for this point.

Chikungunya infection can also induce hemorrhagic complications (*11*). Overall, 59 infected mothers reported epistaxis or gingivorrhagia, but these symptoms are frequent in pregnant women. We found no difference in the risk for vaginal bleeding during pregnancy or for third-stage hemorrhage.

We observed more congenital malformations in babies exposed to chikungunya in utero than in unexposed babies (19 vs. 15). However, this difference was not significant and we could not reach a definitive conclusion for this factor because only 99 women in our sample had a chikungunya infection during the first trimester. It would have required 1,340 children in each group to show a doubling of the risk (4% vs. 2%) with a power of 80% ($\beta = 0.20$ and $\alpha = 0.05$). There is no information on long-term consequences of in utero exposure to chikungunya. Some newborns in our cohort were followed up until the age of 2 years. Analyses are underway to assess long-term consequences.

Chikungunya infection was more frequent in women with a lower educational level. That disadvantaged populations are overexposed to transmissible infectious diseases, including dengue and chikungunya, has been shown (*26*,*27*). Therefore, during outbreaks, information and protection for all pregnant women should particularly be emphasized, especially for those whose educational level may result in a lack of basic knowledge about disease prevention. It might be useful to screen these women actively and conduct home visits to verify application of basic antivector measures (destruction of mosquito breeding sites and larval havens around the home, wearing of long-sleeved clothing, and use of repellents appropriate for pregnant women and of mosquito netting).

The chikungunya vector (*Ae*. *albopictus*) is found in Asia, Oceania, North and South America, and Europe. International travel creates the possibility of large-scale epidemics in countries previously considered free of chikungunya (*16*,*28*). An epidemic of chikungunya was observed in a temperate zone (Italy) in 2007 (*18*). Our results will provide information for pregnant women in unimmunized populations during epidemics.

Acknowledgments

We thank the patients for participating in the study; the healthcare staff at the participating hospitals for accepting an additional workload during the outbreak; Jo Ann Cahn for editorial assistance; and Georges Barau, Vanessa Basque, Emmanuelle Bessueille, Anne-Sophie Charpentier, Daniel Daguindeau, François Favier, Marc Gabriele, Jean-François Grandjean, Philippe Grivard, Annie Lagarde, Joëlle Perrau, Annie Naty, Hanitra Randrianaivo, Jean-Pierre Rivière, Martine Robillard, Pierre-Yves Robillard, Jean-Claude Sommer, Sandrine Terrentroy, Yasmina Touret, and Jacques Tuaillon for helping with patient participation.

This study was supported by the French Ministry of Health as part of the Hospital Clinical Research Plan for 2006.

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Effects of Mumps Outbreak in Hospital, Chicago, Illinois, USA, 2006

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In 2006, nearly 6,000 mumps cases were reported in the United States, 795 of which occurred in Illinois. In Chicago, 1 healthcare institution experienced ongoing transmission for 4 weeks. This study examines the outbreak epidemiology and quantifies the financial affect on this organization. This retrospective cohort study was conducted through case and exposure identification, interviews, medical record reviews, and immunologic testing of blood specimens. Nine mumps cases resulted in 339 exposures, 325 (98%) among employees. During initial investigation, 186 (57%) of the exposed employees had evidence of mumps immunity. Physicians made up the largest group of noncompliers (55%) with mumps immunity testing. The cost to the institution was $262,788 or $29,199 per mumps case. The outbreak resulted in substantial staffing and financial challenges for the institution that may have been minimized with readily accessible electronic employee vaccination records and adherence to infection control recommendations.

Mumps, a highly contagious illness caused by a paramyxovirus, causes influenza-like symptoms and salivary gland swelling. Although rare, complications may include encephalitis, meningitis, orchitis, and oophoritis. The virus replicates within the upper respiratory tract and is transmitted through direct contact with respiratory droplets or saliva and through fomites. The incubation period ranges from 12 to 25 days; persons who contract mumps are considered infectious from 3 days before symptoms appear through 9 days after symptoms appear. Although no specific treatment exists, the disease is preventable through use of measles, mumps, rubella (MMR) vaccine usually provided to children ≈1 year of age with a booster dose administered before children start school. Clinical diagnosis is confirmed by laboratory testing that includes culture, serologic analysis, or real-time reverse transcription–PCR (RT-PCR) (*1*,*2*).

During January 1–October 7, 2006, 45 states reported 5,783 confirmed or probable cases to the Centers for Disease Control and Prevention (CDC). Six states, including Illinois, were responsible for 84% of reported cases. Mumps is generally more common among unvaccinated children, but nationally this outbreak occurred primarily among college-age persons (*3*). In Chicago, reported mumps cases began to increase in March 2006. By the end of the year, the Chicago Department of Public Health had 73 confirmed and probable cases. More of these cases were in an older age group (20–29 years) than was nationally observed (*4*).

Most healthcare worker (HCW) cases were concentrated in 1 hospital, Northwestern Memorial Hospital (NMH), Chicago Illinois, USA, which experienced ongoing transmission during April 23–May 23, 2006. The situation created resource and economic challenges to the organization. We examine the control and effects of this outbreak in a tertiary care center.

Methods

Clinical Setting and Patient Population

NMH is an 825-bed academic medical center. All adult patient care rooms are single occupancy; the neonatal intensive care unit (ICU) is multiple occupancy with 8 nurseries each housing 4–12 isolettes (self-contained incubator units, total of 67 isolettes). The patient cohort comprised all mumps case-patients and persons exposed to them at NMH

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DOI: 10.3201/eid1603.090198

during April 23–May 23, 2006. The NMH Institutional Review Board approved this study.

Definitions

According to CDC, a clinical case of mumps is defined as acute onset of unilateral or bilateral tender, self-limited swelling of the parotid or other salivary glands lasting ≥2 days without other apparent cause. Confirmed cases are either laboratory confirmed or meet the clinical case definition and are epidemiologically linked to a confirmed or probable case. Probable cases meet the clinical case definition but are neither laboratory confirmed nor epidemiologically linked to another confirmed or probable case. Two probable cases that are epidemiologically linked are considered confirmed, even in the absence of laboratory confirmation (*3*,*5*). An exposure is defined as being within 3 feet of a person with mumps without use of appropriate personal protective equipment (*6*). A close contact is a visitor or family member exposed to a person with a confirmed or probable case. Persons are considered to have mumps immunity if they have documentation of receipt of 2 doses of mumps-containing vaccine, positive mumps immunoglobulin (Ig) G serologic results, or documentation of physician-diagnosed mumps (*4*,*7*). Persons with mumps serologies in the indeterminate range are considered nonimmune.

Outbreak Investigation

The NMH Infection Control and Prevention Department (IC) investigated all mumps cases in an attempt to identify the index case and all persons who were exposed. Case-patients were placed in airborne infection isolation, as were exposed, nonimmune patients during their incubation period. Upon hospital discharge, case-patients and exposed patients were instructed to follow-up with the NMH Infectious Diseases Clinic (ID) or their primary care physicians. Similarly, patients discharged before recognition of exposure were contacted and referred to either ID or their primary care physician. IC sometimes needed to assign a provisional case status and to recommend a disposition before laboratory results were known. All cases were reported to the jurisdictional local health departments, and NMH provisional case status was retrospectively compared with the final case status assigned by the health departments.

According to NMH policy, all employees with communicable work-related illnesses or exposures are evaluated in the Corporate Health Department (CH). During this outbreak, employees with illnesses consistent with mumps were evaluated, furloughed through day 9 of their illness, and cleared by CH before returning to work. Ill employees were paid either through Workers' Compensation (WC) after the first 3 days, for which employees are required by the Illinois State Workers' Compensation Commission to use personal time off, or through the Short Term Injury and Illness Plan. Exposed employees were paid through a furlough account established by NMH during days 12–25 of the incubation period if nonimmune or while awaiting serologic test results. Employee compensation was managed through the NMH WC and Human Resources (HR) departments. Close contacts were referred to ID where immunity was determined at no charge to them.

Infection control data were collected through interviews and medical record review. Patient data were obtained from electronic medical records and employee data from written medical charts. Data included name, job title for employees; hospital location; exposure source for cases; and immunologic status, including previous receipt of MMR vaccine, history of mumps, and mumps serologic result with laboratory test date.

Vaccine Program

Before 2003, only measles and rubella vaccination were routinely recorded in employee health records; thus, mumps vaccination status was often unavailable. To quickly assess mumps immunity during this outbreak, an intranet survey was created (SurveyMonkey, Portland, OR, USA) and made available to all employees. CH personnel reviewed survey results; results were not corroborated during the outbreak because of time constraints. To facilitate evaluation, counseling, and vaccination, nonimmune employees were seen either in CH, the Northwestern Medical Faculty Foundation Travel Medicine and Immunization Center, or in 1 of 2 satellite clinics established for this outbreak. Staff were classified as either high-risk caregivers (HRCs), low-risk caregivers (LRCs) or noncaregivers (NCs) to allow vaccine prioritization. HRCs were those who worked in areas where mumps cases were located or worked with pregnant or immunosuppressed patients. LRCs were persons who cared for patients in other inpatient or outpatient areas. To conserve resources, NCs were encouraged to seek evaluation with their primary care physicians but were not turned away if they sought evaluation at an NMH location.

Laboratory Evaluation

NMH's Immunology Laboratory performs mumps qualitative IgG antibody testing. Although most tests were performed in house, because of a low manufacturer's supply of test kits, patient IgG testing was sent to a reference laboratory, and in-house testing was reserved for employees who were within 4 days of furlough. Turnaround time for the in-house test was decreased from 72 to 24 hours, and staffing was increased on weekends throughout the outbreak to ensure timeliness of test results. Reference laboratory turnaround time was 1–3 days. NMH's Referred Testing Department sent serum to a reference laboratory

for IgM and IgG antibody testing and buccal swabs to the Illinois Department of Public Health for RT-PCR.

Financial Effects and Data Analysis

The financial effects were determined by tabulating the cost of personnel assistance and resource use. The cost of personnel assistance (i.e., lost productivity) was systematically provided by departmental leaders after the investigation. A total dollar value was assigned each department by estimating the time spent by each employee on outbreak and exposure management. The cost for resources, represented by exact dollar amounts, includes medical evaluations, vaccines, laboratory evaluations, and employee compensation. Data for personnel assistance were stratified by department and aggregated to provide a total estimate. Data for resources were stratified by type of activity and aggregated to provide a total cost. Additionally, an estimate of the cost of maintaining a routine 2-dose MMR vaccination program and adequate employee medical records was calculated to compare with the cost of the outbreak. Data from 2008 were used for this calculation because 2008 was the first year NMH had complete financial records for the 2-dose MMR vaccination program. Financial data are rounded to the nearest dollar amount.

Results

Outbreak Investigation

Nine mumps cases occurred at NMH, 7 among employees and 2 among inpatients (Table 1). Six were primary and 3 were secondary cases (Figure 1). Eight cases were symptomatic. Eight case-patients were women; the average age of all case-patients was 34 years (range 26–39 years). Two had documented receipt of 2 MMR vaccines, 2 had positive IgM serologic results, and none had documentation of prior mumps infection. Retrospectively, jurisdictional health departments assigned case status as follows: 4 confirmed, 3 probable, and 2 that could not be confirmed because even though both had clinical symptoms, 1 had negative laboratory results and the other had no known history of exposure.

During the outbreak, 339 persons (325 employees and 14 close contacts) were reported as having been exposed to a person with mumps, resulting in an average of 38 exposures per case (Figure 2). Of the 325 employees, 186 (57%) were deemed immune: 16 (9%) with documented physician-diagnosed mumps, 14 (7%) with documented receipt of 2 doses of mumps-containing vaccine, and 156 (84%) with prior laboratory evidence of immunity. None of these employees required time off work because of the timely reporting of their mumps immune status. The remaining 139 (43%) employees required laboratory testing for immunity. Of these, 63 (45%) underwent testing, with serologic results as follows: 33 (52%) positive, 11 (18%) equivocal, and 19 (30%) negative. Overall, 219 (88%) of the 249 HCWs evaluated were immune to mumps. The remaining 76 (55%) employees who required testing for mumps immunity did not comply with CH evaluation (Figure 3). Of these persons, physicians made up 55%; registered nurses (RNs), 29%; unit staff, 13%; and nonunit staff, 3%. Fourteen close contacts required laboratory testing for mumps immunity, and all were immune.

A total of 59 employees were absent from work for 282 days for reasons that included having mumps, being nonimmune, and awaiting symptom evaluation or laboratory test results (Table 2). Employee time off work ranged from 1 to 24 days (average 5 days). RNs accounted for most of the work absences (n = 25, 42%) and took off the

Table 1. Epidemiology of 9 mumps cases, Northwestern Memorial Hospital, Chicago, Illinois, USA, 2006*

Case no.	Patient age, y/sex	No. MMR vaccine doses received	Clinical signs	Date of symptom onset	Serologic test results		DoH case disposition
					IgM	IgG	
1	35/F	1	Fatigue, unilateral facial swelling	Apr 24	–	+	Not a case
2	30/F	0	Fever, chills, stiff neck, bilateral facial swelling	May 5	–	+	Not a case
3	26/F	2	Fatigue, fever, headache, stiff neck, bilateral facial swelling	May 12	IND	+	Probable
4	39/F	0	Headache, sore throat, stiff neck, myalgias, bilateral facial swelling	May 15	+	IND	Confirmed
5	38/F	0	Flu-like illness, bilateral facial swelling	May 19	–	+	Probable
6	30/F	0	Headache, sore throat, myalgias, tender submandibular nodes	May 19	–	+	Probable
7	26/F	2	Sore throat, headache, bilateral facial swelling	May 22	–	+	Confirmed
8	44/F	1	Bilateral facial swelling	May 22	–	+	Confirmed
9	35/M	0	Asymptomatic	May 23	+	+	Confirmed

*MMR, measles, mumps, rubella; Ig, immunoglobulin; DoH, Department of Health; –, negative; +, positive; IND, indeterminate.

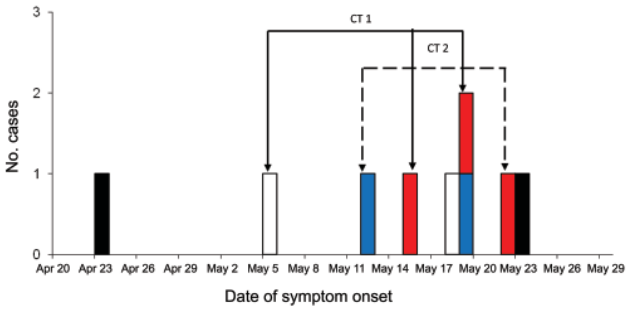

Figure 1. Epidemiology of 9 mumps cases at Northwestern Memorial Hospital, Chicago, Illinois, USA, April 23–May 23, 2006. Black bars, community-acquired cases among staff members; white bars, community-acquired cases among patients; red bars, secondary cases among staff members; blue bars, presumed work-related cases among staff members; CT1, first chain of transmission; CT2, second chain of transmission.

most days (94 days, 33%), followed by resident physicians (49 days, 17%). Furlough was the most used type of time off, with 229 days (81%), primarily for nonimmunity (178 days, 78%) followed by furlough awaiting serologic test results (51 days, 22%).

During April 1–June 31, 2006, 416 mumps IgG serologies were performed at NMH; 58 IgM and 207 IgG serologic results were sent to a reference laboratory. Twenty-nine buccal swabs were sent to the Illinois State Laboratory for mumps RT-PCR, and only 2 were positive, both from outpatients unrelated to the institutional outbreak.

Vaccine Program

Of the 6,600 NMH employees, 5,150 (78%) completed the intranet survey to assess their mumps immunity (Figure 4). Of these, 1,560 (30%) were HRCs and 3,590 (70%) were LRCs or NCs. Ninety-one percent of HRCs and 74% of LRCs and NCs completed the survey. Of the HRCs who completed the survey, 699 (45%) required additional follow-up; however, only 355 (51%) complied. Of those who complied, 228 (64%) received vaccination. In comparison, 1,072 (30%) LRCs and NCs required additional follow-up, and 386 (36%) complied. Of these, 223 (58%) received vaccination. Overall, 127 (36%) of HRCs and 163 (42%) of LRCs or NCs either declined or did not require vaccination. The average time for employee evaluation in CH was 30–45 minutes, and the 2 satellite clinics operated for 177 hours. From April 20 through September 1, 2006, CH administered a total of 755 MMR vaccinations, 451 to survey participants.

Financial Effects

The estimated cost of personnel assistance during the mumps outbreak was $66,432, led by IC at $36,746 (55%) (Table 3). The largest contribution from a hospital unit was the neonatal ICU at $6,624 (10%). The actual cost of resources was $196,356. The largest resource contributors were HR resulting from compensation for employee time off work at $91,318 (47%) and CH at $56,256 (27%) from time required for medical record review. The total cost of the outbreak was $262,788, representing a 3:1 ratio of resource to personnel costs. Cost per mumps case was $29,199.

In comparison, in 2008 maintaining a routine 2-dose MMR vaccination program and adequate employee medical records cost ≈$66,025. This figure represents the annual number of new employees (n = 978), all of whom required a $30 medical record review and the annual number of MMR vaccinations given (n = 667) at $55 each. Thus, the cost of controlling the mumps outbreak was 4× the cost of maintaining a routine MMR prevention program.

Discussion

Transmission of mumps can occur within hospitals, but outbreaks with secondary transmission such as the one at NMH are rarely reported (*8*,*9*). One of the most widely reported incidents of nosocomial transmission occurred during a community mumps outbreak in Tennessee in 1986–1987 (*8*). Although only a small number of cases were nosocomially transmitted, this in-hospital outbreak illustrates the threat that mumps and other illnesses can pose to patients and HCWs (*8*,*10*).

Although investigators have quantified the impact of nosocomial mumps outbreaks, in-depth analysis of resource use during a large-scale nosocomial mumps outbreak has not been published (*9*,*11*). Analysis of this outbreak assigned a cost for the resources used and the personnel affected. Most of the resource cost was attributable to HR

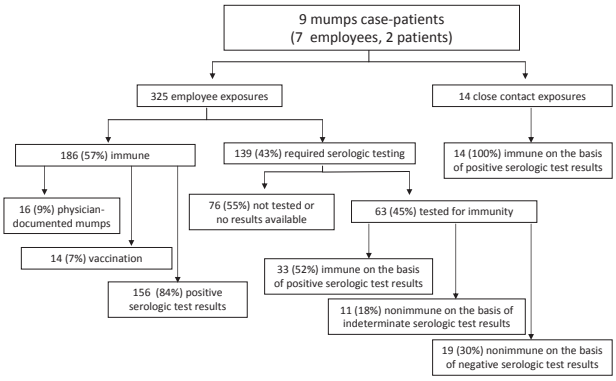

Figure 2. Immune status results among employees and close contacts exposed to 9 persons who had mumps, Northwestern Memorial Hospital, Chicago, Illinois, USA, 2006. For those deemed immune, immunity is grouped based on historical documentation of serologic status, mumps infection, or vaccination. All others were required to report for serologic testing during the outbreak; for those who complied with the required testing, immune status is provided.

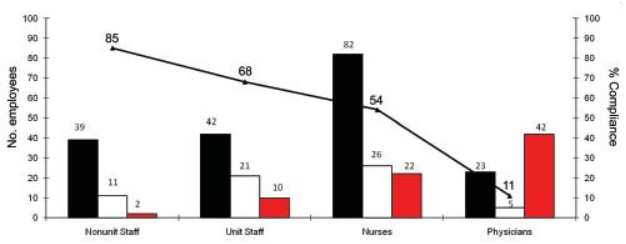

Figure 3. Mumps immunity status and compliance among employees, Northwestern Memorial Hospital, Chicago, Illinois, USA, 2006. Black bars, no. employees with history of immunity; white bars, no. employees who complied with required antibody titer testing; red bars, no. employees who did not comply with required antibody titer testing; black line, percentage of employees in compliance. Unit staff consisted of nurse managers, secretaries, patient care technicians, clinical coordinators, and emergency department assistants; nonunit staff consisted of applications analysts, counselors, radiographers, resource coordinators, respiratory therapists, records specialists, safety technicians, patient escorts, housekeeping workers, and food services workers.

for compensation for staff work absences and to CH for employee health record review. Additionally, personnel most affected were from IC and from the neonatal ICU, the inpatient unit requiring the most staffing substitutions. In comparison, a nosocomial mumps outbreak in Utah in 1996 reported the total cost of the outbreak in an inpatient pediatric facility was $3,140, substantially lower than our cost (*9*). Examination of these 2 outbreaks, however, indicates that they are not comparable. The Utah facility was much smaller than NMH (45 vs 825 beds), had fewer staff, and had only 2 cases. The smaller work environment and magnitude of the outbreak posed less opportunity for exposure to an infected person and required far fewer resources for outbreak control. In contrast, a neonatal ICU outbreak of infection with respiratory syncytial virus, an illness spread through a similar route, involving 9 infants was reported to have cost >$1.15 million (*12*). Although the number of cases is similar to ours, the increased cost of the outbreak of infection with respiratory syncytial virus reflects the need for intensive care and expensive postexposure prophylaxis (*12*). These discrepancies highlight the need for organizations to conduct and report detailed disease-specific analyses to assist similar institutions planning for resource use during outbreak prevention and control.

At NMH, the lack of complete and easily retrievable employee health records contributed substantially to the overall outbreak cost. Until recently, only documentation of rubella and measles immunity was required and mumps immune status was often not recorded; additionally, vaccination information was not available electronically. During the outbreak, the need to rapidly evaluate the mumps immunity of our workforce would have required review of >6,000 employee health records, a task not deemed practical to prevent ongoing disease transmission and excessive employee furlough. This challenge led to development of an electronic survey to query employees about their mumps immunity. Although obviously suboptimal, this approach allowed CH to focus on record review for employees who either did not know their status or did not respond to the survey and to manage the ongoing vaccine campaign. This situation is not unique to NMH. Analysis of previous mumps outbreaks identified complete and easily retrievable employee vaccination records as an integral step in reducing the resource and financial costs to the hospital (*8*,*9*,*13*,*14*). If employee health information was complete and accessible, more than one fourth of our outbreak cost might have been averted.

Vaccination of HCWs is vital to mumps outbreak prevention. Although numerous outbreaks have occurred in populations with only 1-dose vaccine coverage, the national mumps outbreak of 2006 occurred during the highest 2-dose vaccine coverage in the United States at 87%, making this the first large-scale national mumps outbreak associated with 2-dose vaccine failure. The estimated herd immunity threshold for mumps ranges from 88% to 92%, and during the outbreak at NMH, 88% of our evaluated workforce reported mumps immunity. The experience nationally and at our institution supports the concept that an increased level of group-specific immunity may be required to prevent transmission in settings in which close or prolonged contact occurs, particularly in crowded conditions, such as those within healthcare institutions (*9*,*13*,*15*). The possibility of vaccine failure highlights the need to maximize immunity among HCWs with 2 doses

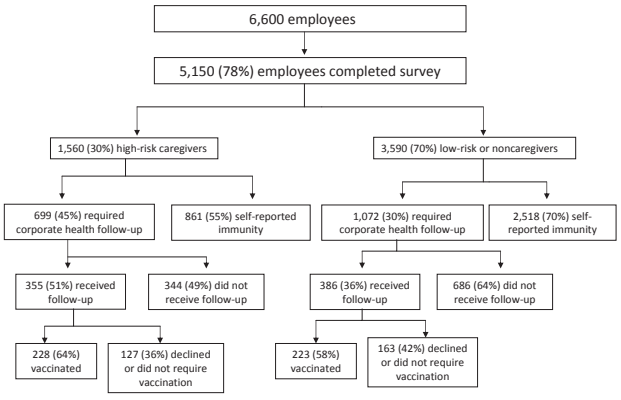

Figure 4. Survey results of self-reported mumps immunity among workforce, Northwestern Memorial Hospital, Chicago, Illinois, USA, 2006. Results are categorized by high-risk caregivers, those who worked in areas where mumps cases were located or worked with pregnant or immunosuppressed patient populations; low-risk caregivers, those who cared for patients in other inpatient or outpatient areas; or noncaregivers. Compliance with corporate health evaluation and vaccination for those who did not report immunity are also shown.

Table 2. Characteristics of employee work absences during mumps outbreak, Northwestern Memorial Hospital, Chicago, Illinois, USA, 2006*

Type of time off	Job title	No. employees	Days allowed
PTO	Registered nurse	1	3
	Patient escort	1	2
Short-term injury and illness plan	Unit secretary	1	6
WC	Patient care technician	1	24
	Registered nurse	1	10
WC and PTO	Registered nurse	2	8
Furlough			
Because of nonimmunity	Registered nurse	9	48
	Physician	5	49
	Unit staff†	9	42
	Nonunit staff‡	7	39
Because of pending titer results	Registered nurse	12	25
	Unit staff	8	20
	Nonunit staff	2	6
Total		59	282

*PTO, personal time off; WC, workers' compensation
†Nurse managers, secretaries, patient care technicians, clinical coordinators, emergency department assistants.
‡Applications analyst, counselor, radiographer, resource coordinator, respiratory therapist, records specialist, safety technician, patient escort, environmental services, food services.

of MMR vaccine and to address the age of administration of an MMR booster or the addition of a third vaccine dose to prevent future outbreaks (*13*–*15*).

Our outbreak highlights the inaccuracies that can exist in mumps case recognition, resulting in both underestimation and overestimation of disease. Cases can be underestimated because patients are contagious for days before symptoms appear, and up to one third of patients never develop symptoms but can still spread disease. Notably, 1 exposed, asymptomatic employee underwent IgG and IgM testing and was positive for IgM. Fortunately, no secondary cases are known to have resulted from exposure to this person. In addition, overestimation can occur when presumptive case status is assigned on the basis of clinical presentation before laboratory results are available. At NMH, 2 probable cases could not be confirmed by the health departments. These cases led to additional exposure evaluations. Although prompt initiation of infection control measures is vital to control a mumps outbreak, investigators should be aware of the challenges in accurate case recognition.

The lack of laboratory resources also increased the cost of the outbreak. The on-site laboratory testing facility required increased staffing to complete timely serologic testing and later had a shortage of testing kits. The need to send specimens to a reference laboratory delayed test results and led to assignment of presumptive case status on the basis of symptoms resulting in potentially unnecessary exposure evaluations. In addition, the hospital had to furlough exposed employees whose immune status was unknown until serologic results were available.

The lack of compliance with IC recommendations for exposure evaluation and vaccination was evident primarily among physicians. This reaction was similar to that during a mumps outbreak in 1987 at the Chicago Mercantile Exchange in which the intense nature and competitiveness of the profession encouraged employees to work while ill (*16*). The reasons for lack of compliance at NMH, particularly among physicians, are unknown, but the urgent nature of the profession is expected to have played a major role. That some employees minimized the risk for exposure or thought the follow-up process was too cumbersome also has been speculated. Another finding was the discovery of a few persons who claimed exposure to benefit from time off work. Cooperation between CH, IC, WC, and HR led to detection and management of these rare instances.

Table 3. Financial effects of mumps outbreak, Northwestern Memorial Hospital, Chicago, Illinois, USA, 2006*

Type of expense and department	Cost, US$*
Personnel	
Human resources	1,066
Infection control and prevention	36,746
Laboratories	7,312
Medical administration	1,500
Nursing units	10,808
Patient escort	1,200
Risk management	300
Environmental and occupational safety	7,500
Total personnel cost	66,432
Resources	
Corporate health	56,256
Human resources employee compensation	91,318
Infectious diseases clinic	1,000
Laboratories	6,842
Travel medicine and immunization center	2,240
Vaccination program	38,700
Total resource cost	196,356
Total cost to hospital	262,788

*Rounded to the nearest dollar.

When examining the types of employee compensation provided, an inherent inequality was established. Ill employees were not fully compensated for their work absence (67% of the employee's average weekly wage after taking 3 days of personal time off). These employees were required to take WC, and the rate of compensation is set according to the Illinois Workers' Compensation Act (www.state.il.us/Agency/IIC/act.pdf). In contrast, exposed employees were compensated by a system specifically established for this outbreak by the hospital because WC will not cover such costs. These persons were compensated 100% of their salary. This unbalanced system of reimbursement may require reevaluation for future outbreaks so that ill persons do not feel penalized or fail to self-disclose illness.

This study has several limitations. First, recall bias may have occurred, particularly when departmental leaders retrospectively estimated personnel costs. Second, the reliability of self-reported information obtained through interviews and the intranet survey regarding mumps immunity was not validated during the outbreak and may have contributed to either overestimation or underestimation of mumps immunity in our workforce. Finally, the findings of this study may not be generalizable because all healthcare institutions are unique environments.

We examined the effects of the 2006 national mumps outbreak within a healthcare institution. Our cost of >$262,000 makes a strong business case for healthcare organizations to improve infectious diseases prevention and control strategies. A comprehensive program that consists of maintaining complete electronic employee health records, identifying cases and employee exposures rapidly, enforcing compliance with infection control recommendations, and developing plans to alleviate laboratory shortages is of paramount importance for outbreak control. Reports of detailed epidemiologic and financial analyses of infectious disease outbreaks can facilitate emergency preparedness and response planning for comparable healthcare organizations.

Acknowledgments

We thank Northwestern Memorial Hospital and Northwestern University Feinberg School of Medicine for providing financial support for this research.

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Blood Meal Analysis to Identify Reservoir Hosts for *Amblyomma americanum* Ticks

Brian F. Allan, Lisa S. Goessling, Gregory A. Storch, and Robert E. Thach

Efforts to identify wildlife reservoirs for tick-borne pathogens are frequently limited by poor understanding of tick–host interactions and potentially transient infectivity of hosts under natural conditions. To identify reservoir hosts for lone star tick (*Amblyomma americanum*)–associated pathogens, we used a novel technology. In field-collected ticks, we used PCR to amplify a portion of the 18S rRNA gene in remnant blood meal DNA. Reverse line blot hybridization with host-specific probes was then used to subsequently detect and identify amplified DNA. Although several other taxa of wildlife hosts contribute to tick infection rates, our results confirm that the white-tailed deer (*Odocoileus virginianus*) is a reservoir host for several *A. americanum*–associated pathogens. Identification of host blood meal frequency and reservoir competence can help in determining human infection rates caused by these pathogens.

Zoonotic pathogens, which reside in animal reservoir species and may at times spill over into human populations, are emerging at an unprecedented rate (*1*). Among these pathogens, several vector-borne pathogens have garnered considerable attention for the toll they exact on human health, which a growing body of evidence indicates may be exacerbated by anthropogenic environmental change (*2*–*4*). A rigorous understanding of the transmission dynamics of pathogens from infected wildlife hosts to vector organisms is critical to explorations of the ecology of vector-borne diseases.

Among the most rapidly emerging vector-borne zoonotic pathogens in the United States are several that are transmitted by the lone star tick (*Amblyomma americanum*). These pathogens include *Ehrlichia chaffeensis* and *E. ewingii*, both agents of human ehrlichiosis, and *Borrelia lonestari*, a potential agent of southern tick–associated rash illness (*5*). Ticks generally acquire pathogens by 2 primary modes of transmission: vertical (i.e., transovarial) transmission, whereby the pathogen is acquired maternally during development of the egg, and horizontally, whereby the pathogen is acquired through a blood meal on a reservoir-competent and infectious animal host. Recent research suggests that *E. chaffeensis* and *E. ewingii* are acquired horizontally (*6*,*7*); *B. lonestari* is likely transmitted horizontally and vertically (*8*). Several lines of evidence suggest that white-tailed deer (*Odocoileus virginianus*) are a major reservoir host for all 3 pathogens (*9*). Nonetheless, several other species have also been implicated as potential reservoirs, and our understanding of their relative roles in disease transmission remains incomplete.

Efforts to identify reservoir hosts for vector-borne zoonotic pathogens have historically been labor-intensive exercises, often requiring the capture of potential wildlife hosts, experimental infection with the pathogen of interest, and a subsequent examination of the efficiency with which these hosts pass the infectious agent to vector organisms under controlled conditions (*10*). However, such laboratory-based estimates may fail to capture the true distribution of host reservoir competencies because of unknown consequences of host selection behavior by vector organisms or the unmeasured contributions of cryptic reservoir hosts (*11*). An efficient solution has emerged in the form of host blood meal identification by molecular methods.

Because of the challenges posed by the duration of tick life cycles and host-seeking behavior, the feasibility of host blood meal identification in ticks was only recently established (*12*). Research efforts have converged upon a 2-step process: PCR amplification of and labeling with biotin any

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DOI: 10.3201/eid1603.090911

remnant vertebrate DNA isolated from a tick, and reverse line blot (RLB) hybridization whereby host-specific oligonucleotide probes are used to detect the biotin-labeled amplified host DNA. Several researchers have successfully used this technology to identify the reservoir hosts for numerous pathogens transmitted by *Ixodes ricinus*, a preeminent vector of tick-borne diseases in Europe (*13*–*16*). We describe the development of host-specific probes and the identification of host blood meals in wild-caught nymphal life stage *A. americanum* and present direct estimates of the reservoir capacity (an estimate of the absolute contribution of a reservoir host to the prevalence of infection in a tick population) for white-tailed deer and other reservoir hosts for the emerging *A. americanum*–associated zoonotic pathogens (*17*).

Materials and Methods

Field Collections

Questing *A. americanum* ticks were collected from 5 conservation areas and state parks in and surrounding St. Louis, Missouri, USA, during 2005 and 2007–2008. Ticks were collected either by dragging a 1-m^2 white cloth along the ground and over vegetation or by using CO_2-baited traps, whereby sublimating dry ice was used to attract ticks, which then became ensnared on double-sided carpet tape surrounding the trap. Both methods have proven effective for sampling nymphal and adult life stages of *A. americanum* (*18*). Captured ticks were removed and preserved in 70% ethanol for future identification and molecular analyses. Sampling efforts were limited to deciduous forested areas, which are the primary habitats in which *A. americanum* completes its life cycle (*5*). All subsequent analyses were limited to host-seeking nymphal life stage ticks, which for *A. americanum* are often presumed to have taken only 1 prior blood meal in the larval life stage.

Laboratory Methods

DNA Extraction and Amplification

Nymphal life stage *A. americanum* were identified under a dissecting microscope before DNA extraction using the method of Kierans and Durden (*19*). Ticks were individually processed using 1 of 2 methods. All ticks collected in 2005 and most of those collected in 2007 were processed using the ammonium hydroxide method described previously by Pichon et al. (*13*). The remainder of the 2007 and all of the 2008 ticks were processed using a modified method described by Hammer et al. (*20*). The success of each method of DNA extraction was confirmed by PCR amplification and agarose gel electrophoresis of tick mitochondrial 16S rDNA as described (*21*,*22*).

Bacterial DNA was amplified in a multiplex PCR containing 2 sets of primers. Universal primers 0206 and 0209, previously described by Pichon et al. (*13*), were used to amplify a portion of the 16S rDNA, and primers 23SN2 and 5SCB, described previously by Rijpkema et al. (*23*), were used to amplify the 23S–5S intergenic spacer of the *Borrelia burgdorferi* complex. Primers 0209 and 5SCB were biotin labeled at the 5′ end to enable detection of the amplicons in the RLB assay. Primers were obtained from IDT (Coralville, IA, USA). Each set of amplification reactions contained at least 1 positive control (10 μL of known pathogen DNA) and 1 negative control (10 μL of DNA extraction negative control).

Vertebrate DNA was amplified by PCR using the biotin labeled primer 0049, described by Pichon et al. 2003 (*13*), and primer 0035 (5′-TTCTAGAGCTAATACATGCCRA-3′). These primers amplify a portion of the vertebrate (mammal and reptile) 18S rRNA gene. Primers were obtained from IDT. As with the bacterial DNA amplifications, at least 1 positive control (DNA from vertebrate tissue) and 1 negative control (negative DNA extraction control) were included with each set of PCRs.

Vertebrate Tissue DNA Extraction, Sequencing, and Probe Design

A small piece of vertebrate tissue, generally liver or muscle, was frozen on dry ice and then pulverized. The sample was then prepared using either the ammonium hydroxide or Chelex method. The resulting supernatant was removed to a fresh tube and a dilution of this supernatant was used in the PCRs.

Primers 0066 and 0067 (*13*) were used to amplify a 350–400-bp fragment of the vertebrate 18S rRNA gene. This fragment contains the area amplified by primers 0049 and 0035. Primers were obtained from IDT. PCR products were purified by using the Wizard SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA). The purified amplicons were double-strand sequenced by using primers 0066 and 0067 by the Protein and Nucleic Acid Chemistry Laboratory at Washington University with ABI Prism Dye Terminator BigDye Premix version 1.1 (Applied Biosystems, Foster City, CA, USA).

MegAlign and EditSeq softwares (DNASTAR, Inc., Madison, WI, USA) were used to align and edit sequence data. The obtained sequences were aligned with 18S sequences found in GenBank and areas of variability were used to design probes.

Reverse Line Blot Hybridization

An RLB assay was used to identify bacterial DNA amplified from the tick lysates. In the assay, biotin-labeled PCR products are hybridized against a set of bacteria-specific probes (Table 1) that have been covalently linked to

Table 1. Oligonucleotide sequences of bacterial probes used in reverse line blot assay

Probe ID	Nucleotide sequence (5′ → 3′)	Target organism (rRNA genes)	Reference sequence
Ptg011	AACATGAACATCTAAAAACATAAA	*Borrelia garinii* (23S–5S)	*
Ptg012	AACATTTAAAAAATAAATTCAAGG	*B. afzelii* (23S–5S)	*
Ptg013	CATTAAAAAAATATAAAAAATAAATTTAAGG	*B. valaisiana* (23S–5S)	*
Ptg009	CTTTGACCATATTTTTATCTTCCA	*B. burgdorferi* s.l. (23S—5S)	*
Ptg010	AACACCAATATTTAAAAAACATAA	*B. burgdorferi* s.s. (23S–5S)	*
Ptg003	CGAACTTCTGGGTCAAGAC	*B. burgdorferi* s.l. (16S)	†
Ptg020	AGATAACTACTCTCCGTTTG	*B. lonestari* (16S)	AY166715
Ptg022	TCCTAATAGGGGGAGTC	*Ehrlichia chaffeensis* (16S)	M73222
Ptg023	CTTTTAACAGAGGGAGTCA	*E. ewingii* (16S)	M73227
Ptg024	TCCTAACAGGGGGAGTC	*E. canis/ovina/muris* (16S)	AY394465, AY318946, ABO13009
Ptg007	TGGGGATTTTTTATCTCTGTG	*Anaplasma phagocytophilum* (16S)	†
Ptg021	CTACCACTGACGCTGAT	*Rickettsia rickettsii* (16S)	DQ150694
Ptg027	CTTCGGAACGCAGTGAC	*Francisella tularensis* + *F. philomiragia* (16S)	Z21932, Z21933
Ptg026	CTTGGGGAGGACGTTAC	*F. tularensis* subsp. *tularensis* (16S)	Z21932
Ptg029	GCCTATRAGTTAATAGCTTGT	*F. philomiragia* (16S)	Z21933
Ptg028	TCCTGCGATCTTTCTAGA	*F. endosymbiont* of Dv (16S)	AF166256
Ptg032	CATCCAGGGAAGTAAGC	*Arsenophonus* spp. (16S)	AY265347
Ptg030	GCTACAACTGACACTGATG	*R. endosymbiont* of Dv (16S)	AY375427
Ptg031	TACAACTGACGCTAATGC	*R. amblyommii* + *Rickettsia* sp. (16S)	U11012
Ptg035	TCGGAAGATTATCTTTCGG	*R. amblyommii* (16S)	U11012

*Designed by Rijpkema et al. 1995 (*23*).
†Designed by Pichon et al. 2003 (*13*).

an activated Biodyne C membrane (Pall, Ann Arbor, MI, USA) by their 5′ amino group. Our method is based on RLB techniques previously described (*13*,*23*).

The probes were applied in lines to an activated membrane using a Miniblotter 45 (Immunetics, Cambridge, MA, USA). The membrane was stored at 4°C until use. Before starting the hybridization, the membrane was incubated in hybridization buffer (0.3 mol/L sodium chloride, 0.02 mol/L sodium phosphate buffer, 0.002 mol/L EDTA, 0.1% sodium dodecyl sulfate) for 45 min at 42°C. For the hybridization step, the membrane was placed in the Miniblotter with the orientation shifted 90° so that the probe lanes were aligned perpendicular to the slots. Each slot was filled with 140 μL of denatured biotinylated PCR products (10 μL PCR products in 140 μL hybridization solution, heated at 99°C for 10 min, then cooled on ice) and incubated at 42°C for 90 min. The PCR solutions were aspirated off and the membrane was washed twice with hybridization buffer at room temperature, then twice at 50°C with preheated buffer. Biotin-labeled PCR products hybridized to probes were detected using the CDP-Star Universal Detection Kit (Sigma, St. Louis, MO, USA) and exposure to Blue Ultra Autorad film (ISC BioExpress, Kaysville, UT, USA).

A second RLB assay using host specific probes was used to identify vertebrate DNA amplified from the tick lysates (Table 2). The protocol for the vertebrate RLB was the same as for the bacterial RLB except the prehybridization wash, hybridization and high stringency wash steps were all conducted at 62°C.

Tick Identification

To confirm correct identification of *A. americanum* nymphs used in our study, we selected 4 tick samples for which we amplified and then double-strand sequenced a portion of the tick 16S rRNA gene. The 16S+1 and 16S-2 primers described in Black and Piesman (*21*) were used for PCR amplification and sequencing.

Statistical Analyses

All statistics were calculated using Poptools version 3.0 in Microsoft Excel (Microsoft, Redmond, WA, USA) (*24*). We used χ^2 tests with the Yates continuity correction to analyze patterns of pathogen co-infection and the distributions of blood meals among hosts. We estimated 95% confidence intervals for our estimates of reservoir capacity based upon identifiable blood meals using the Wilson score method without continuity correction.

Results

Pathogen Detection

Three of the most widely reported pathogens associated with *A. americanum* (*E. chaffeensis*, *E. ewingii,* and *B. lonestari*) were detected among collections from ≥3 of 5 study sites (i.e., each pathogen was detected from ticks collected at ≥3 locations). Of the 1,383 nymphal life stage *A. americanum* ticks tested, 19 (1.4%) contained *E. chaffeensis*, 31 (2.2%) contained *E. ewingii*, and 18 (1.3%) contained *B. lonestari*. No co-infections with >1 pathogen were detected in any tick. However, χ^2 analyses for each

Table 2. Oligonucleotide sequences of vertebrate probes used in reverse line blot assay*

Probe ID	Probe name	Nucleotide sequence (5′ → 3′)	Reference sequence
PRNA010	Aves	CCGACCTCCGGGGACGC	*
PRNA012	Passeriformes	GGGCCCGCCCGGCAGCT	*
PRNA029	Galliformes	GGGCTCGCCCGGCGGCT	*
PRNA042	Squamata/testudines	CGCTGACCTCCGGGGATGC	*Sceloporus undulatus* (M36359, M59400), *Crotaphytus collaris collaris*† (FJ797666), *Trachemys scripta*† (FJ797668), *Scincella lateralis* (AY217908), *Eumeces fasciatus* (AY217920), *Elaphe obsoleta*† (FJ797667), *Heterodon platirhinos* (M59392)
PRNA043	Amphibia	CGCTGACCCCCAGGGATGC	*Rana amurensis* (AF542043), *R. chensinensis* (AY145522), *Xenopus laevis* (X04025)
PRNA045	Ruminantia	GGTCAGCCTCCTCCCGGC	*Odocoileus* virginianus† (FJ797665), *Capreolus capreolus* L (AY150545), *Cervus elaphus* L (AY150547), *Bos taurus* (AY779625)
PRNA018	Leporidae	CGGGGGGGTGGGCGCCG	*
PRNA047	Leporidae/carnivore	GGTCAGCCTCCCCCCGGC	*Sylvilagus floridanus* (FJ797663), *Procyon* lotor† (FJ797659), *Felis catus* L (AY150542),
PRNA046	Canidae	GGTCAGCCTCCCTCCGGC	*Canis latrans*† (FJ797662), *Canis lupus familiaris*† (FJ797658), *C. lupus familiaris* (DQ287955), *Vulpes vulpes* (AY150549)
PRNA026	Sciurus	CGGTCAGCTTCCCCCCGG	*
PRNA037	Blarina	AGCCTCCCCTCGGCTCCG	*Blarina* sp.† (FJ797661)
PRNA030	Erinaceus	CTCCCTCCGGCTCCGGC	*
PRNA017	Myodes 1	GAGCTCCCCCGCGGCCC	*
PRNA050	Myodes 2	CGACGGGCGCCGACCCC	*Myodes glareolus* (AY150543)
PRNA011	Murinae/gerbilinae	CCCTCCCGGCTCCGGCCG	*
PRNA034	Rattus	CGGTCAGCCCCCTCCCGG	*Rattus norvegicus* (X01117)
PRNA033	Mus	CCGGTGAGCTCCCTCCCGG	*Mus musculus* (X00686)
PRNA035	Sigmodontinae	TCAGCTCCCTCCCGGCCCC	*Peromyscus* sp.† (FJ797660), *Peromyscus leucopus* (AY591913)
PRNA032	Didelphis	CGGCGGCTTCCCCCTAACC	*Didelphis virginiana* (J311677)
PRNA048	Mephitis	GGTCAGCCTCTCCCCGGC	*Mephitis mephitis*† (FJ797664)

*Designed by Pichon et al. 2003 (*13*).
†Sequence obtained in this study.

pair of pathogens indicated that this outcome did not differ from random chance (*E. chaffeensis* and *E. ewingii*: χ^2 = 0.013, df = 1, p = 0.908; *E. ewingii* and *B. lonestari*: χ^2 = 0.024, df = 1, p = 0.877; *B. lonestari* and *E. chaffeensis*: χ^2 = 0.359, df = 1, p = 0.549).

Host Probes

DNA from 13 vertebrate species (for which sequences of 18S rDNA were not available in the GenBank database) were purified and subsequently amplified for sequencing. The amplicons were double-strand sequenced and these sequences together with those available in the GenBank database were aligned to generate vertebrate host probes (Table 2). Eventually, 20 host probes were established, and 34 vertebrate species that were identified from the literature as potentially important hosts were correctly identified to the matching host probe, with 1 exception (*Tamias striatus* reacted with Canidae probe) (Table 3).

Detection of Host DNA

Purified lysates from all 1,383 nymphal life stage *A. americanum* screened for pathogenic microbes in the previous analyses were also subjected to host blood meal identification. Remnant host DNA from 869 (62.8%) of these ticks hybridized with 10 of the 20 host probes used (Table 4). Of these samples, 389 (44.8%) hybridized to the Ruminantia probe, which for wildlife hosts in the St. Louis, Missouri, region is likely limited to white-tailed deer (Table 3). The remaining blood meals were distributed across a variety of taxa. DNA from more than 1 host was detected in 141 nymphal life-stage ticks (Table 4).

Of the 68 *A. americanum* nymphs containing pathogenic bacteria, 47 (69.1%) contained identifiable vertebrate DNA (i.e., that hybridized with ≥1 host probe; Table 5). Of the 15 *E. chaffeensis*–positive samples that contained identifiable vertebrate DNA, 8 hybridized only with the Ruminantia probe, and 4 others hybridized with the Ruminantia probe plus ≥1 additional probes; thus 12 of 15 identifiable samples hybridized with the Ruminantia probe. The other identifiable *E. chaffeensis*–positive samples hybridized either with the Sciurus (n = 2) or the Leporidae (n = 1) probes. For the 23 identifiable *E. ewingii*–positive samples, 12 contained DNA that hybridized only with the Ruminantia probe, 3 that hybridized only with the Sciurus probe, and 1 that hybridized only with the Leporidae probe. All 6 of the identifiable mixed blood meal DNAs hybridized with ≥2 of these 3 host

Table 3. Hybridization by host DNA to vertebrate reverse line blot probes

Probe ID	Probe name	Vertebrate DNA hybridized
PRNA010	Aves	*Turdus migratorius, Meleagris gallopavo, Gallus gallus, Chen caerulescens*
PRNA012	Passeriformes	*T. migratorius*
PRNA029	Galliformes	*M. gallopavo, G. gallus, C. caerulescens*
PRNA042	Squamata/testudines	*Crotophytus collaris, Elaphe obsoleta, Trachemys scripta elegans*
PRNA043	Amphibia	*Rana clamitans*
PRNA045	Ruminantia	*Odocoileus virginianus, Cervus elephus, Bos taurus, Sus scrofa domestica*
PRNA018	Leporidae	*Sylvilagus floridanus, Sus scrofa domestica*
PRNA047	Leporidae/carnivora	*S. floridanus, Felis catus, Procyon lotor*
PRNA046	Canidae	*Canis lupus familiaris, C. latrans, Vulpes vulpes, Tamias striatus**
PRNA026	Sciurus	*Sciurus carolinensis, Sciurus niger, S. griseus, Marmota monax*
PRNA037	Blarina	*Blarina brevicauda, Sorex vagrans*
PRNA030	Erinaceus	No hybridization with any vertebrate DNA tested
PRNA017	Myodes 1	*Myodes gapperi*
PRNA050	Myodes 2	*M. gapperi, Microtus californicus*
PRNA011	Murinae/gerbilinae	*Rattus norvegicus, Mus musculus, Zapus hudsonius*
PRNA034	Rattus	*Rattus norvegicus*
PRNA033	Mus	*M. musculus*
PRNA035	Sigmodontinae	*Peromyscus* spp., *Neotoma fuscipes*
PRNA032	Didelphis	*Didelphis virginiana*
PRNA048	Mephitis	*Mephitis mephitis*

*The reaction was confirmed by using 2 tissue samples. The PCR amplicon was sequenced and matches the Canidae probe.

probes. The remaining identifiable *E. ewingii*–positive sample hybridized only with the Passeriformes probe. For the 9 identifiable *B. lonestari*–positive samples, 4 hybridized with the Ruminantia probe, 1 hybridized with the Sciurus probe, 1 hybridized with the Passeriformes probe, and 1 hybridized with the Squamata/Testudines probe (which is expected to detect DNA from lizards, snakes, and turtles).

Because there is evidence that *B. lonestari* can be transovarially transmitted (*8*), it is crucial to test whether the associations between host blood meals and pathogen infections differ from a distribution expected by random chance alone. The frequency of association between *B. lonestari* infection and the Ruminantia probe ($\chi^2 = 0.033$, df = 1, p = 0.855), the Sciurus probe ($\chi^2 = 0.217$, df = 1, p = 0.641), the Passeriformes probe ($\chi^2 = 0.209$, df = 1, p = 0.647), and the Squamata/Testudines probe ($\chi^2 = 0.639$, df = 1, p = 0.424) did not differ from a distribution expected by random chance. Owing to the detection of host blood meals in pathogen-positive and pathogen-negative ticks, we were able to generate estimates of reservoir capacity (calculated as the proportion of blood meals from a given host that result in an infection for a given pathogen and includes the end products of tick feeding and molting success) for each taxonomic grouping of reservoir host and pathogen species (Table 6).

Tick Identification

Two of the tick samples analyzed contained DNA that reacted with the Squamata/Testudines probe, 1 of which was also positive for *B. lonestari*, and 2 samples contained DNA that reacted with the Passeriformes probe, 1 of which was also positive for *E. ewingii*. The sequences obtained from the 4 ticks were identical except for an extra basepair in 2 of the sequences. The sequences were compared with 16S sequences of other potential tick species in Genbank and had 98%–100% homology with *A.*

Table 4. Identification of host DNA in questing *Amblyomma americanum* nymphs, Missouri, USA, 2005 and 2007–2008

Host data	2005	2007	2008	All
No. nymphs analyzed (no. hosts identified)	75 (33)	489 (240)	819 (596)	1,383 (869)
No. (%) nymphs per identified host				
Ruminantia	5 (15.2)	147 (61.3)	237 (39.8)	389 (44.8)
Galliformes	4 (12.1)	16 (6.7)	77 (12.9)	97 (11.2)
Passeriformes	1 (3.0)	17 (7.1)	76 (12.8)	94 (10.8)
Sciurus	17 (51.5)	13 (5.4)	65 (10.9)	95 (10.9)
Leporidae	3 (9.1)	3 (1.3)	15 (2.5)	21 (2.4)
Squamata/testudines	0	15 (6.3)	3 (0.5)	18 (2.1)
Canidae	0	1 (0.4)	7 (1.1)	8 (0.9)
Leporidae/carnivora	0	0	3 (0.5)	3 (0.3)
Sigmodontinae	1 (3.0)	1 (0.4)	1 (0.2)	3 (0.3)
Mixed	2 (6.1)	27 (11.3)	112 (18.8)	141 (16.2)

Table 5. Blood meal source in pathogen-positive *Amblyomma americanum* nymphs, Missouri, USA, 2005 and 2007–2008

Host	No. *A. americanum* nymphs infected		
	Ehrlichia chaffeensis	*E. ewingii*	*Borrelia lonestari*
Ruminantia	8	12	4
Sciurus	2	3	1
Leporidae	1	1	0
Passeriformes	0	1	1
Squamata/testudines	0	0	1
Mixed	4	6	2
Not identified	4	8	9
Total	19	31	18

americanum sequences, but only 84% homology with *Haemaphysalis leporispalustris* and 81% homology with *A. tuberculatum*.

Discussion

Three of the zoonotic pathogens primarily associated with *A. americanum* (*E. chaffeensis*, *E. ewingii*, and *B. lonestari*) were detected at our field sites at infection rates in nymphal life stage ticks comparable to levels reported elsewhere in the region (*25*,*26*). Our array of host probes indicates that *A. americanum* feed from a variety of vertebrate hosts in the larval life stage, consistent with observations from field studies (*5*). We found that most nymphal *A. americanum* infected with *E. chaffeensis* fed upon a white-tailed deer in the larval stage, consistent with the prevailing hypothesis that this is the major wildlife reservoir for this emerging pathogen (*9*). Analysis of 3 other *E. chaffeensis*-positive blood meals associated with the Sciurus and Leporidae probes suggests that members of the genus *Sciurus* (likely fox and gray squirrels, *S. niger* and *S. carolinensis*, respectively) and eastern cottontail rabbits (*Sylvilagus floridanus*) may also function as wildlife reservoirs for *E. chaffeensis*. Most blood meals detected from *E. ewingii*–positive ticks were also associated with the Ruminantia, Sciurus, or Leporidae probes. Considering the lack of evidence for transovarial transmission of *E. chaffeensis* (*6*) and *E. ewingii* (*7*), we consider the wildlife hosts in these taxa to be the major reservoir hosts in this region.

Table 6. Estimates of reservoir capacity for reservoir hosts of *Amblyomma americanum*–associated zoonoses*

Host	% Bloodmeals associated with pathogen infection (95% CI)	
	Ehrlichia chaffeensis	*E. ewingii*
Ruminantia	2.1 (30 –75.2)	3.1 (33.0–70.8)
Sciurus	2.1 (3.7–37.9)	3.2 (4.5–32.1)
Leporidae	4.8 (1.2–29.8)	4.8 (0.8–21.0)
Passeriformes	0	1.1 (0.8–21.0)
Squamata/testudines	0	0

*CI, confidence interval. *Borrelia lonestari* is omitted because of the confounding influence of transovarial transmission.

No consistent associations between the sources of host blood meals and infection rates with *B. lonestari* in nymphal life stage ticks were found. In light of evidence that *B. lonestari* can be transovarially transmitted (*27*), it may not be possible to determine whether an infected tick acquired this pathogen through a blood meal from an infective host or through vertical transmission from mother to offspring. Therefore, host blood meal identification may not be an adequate means to identify reservoir hosts for this pathogen. Increased samples sizes combined with knowledge of transovarial transmission rates may eventually enable researchers to quantify the contributions of reservoir hosts to infection prevalence of *B. lonestari* in *A. americanum*.

Our data enable us to further generate estimates of reservoir capacity, defined as the absolute contribution of a reservoir host to the prevalence of pathogen infection in a tick population. This metric includes the influence of host abundance, the probability that a host is infected, infectivity of that host, and tick feeding and molting success rates (*17*). Although this metric should not be mistaken for an estimate of actual reservoir competence (i.e., the proportion of ticks that become infected from feeding on infective hosts), it may be more informative because it includes the outcome of several ecologic processes that ultimately determine human risk of exposure to tick-borne pathogens. We found that white-tailed deer do not yield the highest absolute estimates of reservoir capacity for any of the 3 pathogens in our study. However, estimated confidence intervals suggest this outcome may be due to small sample sizes for estimates of reservoir capacity for other reservoir hosts. In light of evidence that white-tailed deer are often infected with these pathogens throughout the range of *A. americanum* ticks (*28*–*30*), we hypothesize that white-tailed deer may be weakly competent reservoirs for these pathogens. However, when taking into account the frequency with which *A. americanum* encounter these abundant hosts, (i.e., reservoir potential) (*31*), it remains apparent that white-tailed deer are major reservoir hosts for *A. americanum*–associated zoonoses.

From the nymphal life stage *A. americanum* that yielded detectable host DNA in this study, 16.2% hybridized with >1 taxonomic probe. Mixed blood meals, presumably caused by bouts of interrupted feeding, have been reported from other studies on ixodid ticks using host blood meal identification, at similar rates to those reported here (*15*,*16*). For example, Morán Cadenas et al. reported multiple host detections from 19.2% of detectable blood meals in *Ixodes ricinus*, with no differences between nymphal and adult life stages (*15*). The absence of a detectable blood meal in 37.2% of the *A. americanum* nymphs examined in our study is also consistent with results from other studies using host blood meal identification in ixodid ticks (*13*–*16*). We speculate that the degradation of remnant host DNA is

the primary cause of this phenomenon, because our ability to detect host blood meals declined later in the season (unpub. data).

It is crucial to temper our conclusions about the role of various hosts derived from our data with some exploration of other factors that may influence the outcome of host blood meal identification. Various factors may influence the detectability of host blood meals, such as the presence of nucleated erythrocytes, host blood volume, permissiveness of hosts (a measure of the ability of a tick to successfully feed to repletion on a given host), and the region of DNA targeted for analysis (*12*). Because the first step of the PCR in our study is subject to dominant template bias, remnant DNA from nucleated erythrocytes may mask mammalian DNA present in mixed blood meals. Additionally, we did not directly quantify the sensitivity of our various host probes, although we did attempt to identify host probe concentrations that yielded equivalent reactions. Nonetheless, variation in host probe sensitivity may introduce another source of error in our findings. In light of these potential limitations to host blood meal identification, field-based studies will remain necessary in order to determine if host blood meal distributions are consistent with the availability of hosts and host-vector interactions.

Host blood meal identification by molecular methods offers a direct and efficient approach for understanding the contributions of both reservoir competent and incompetent hosts to the transmission dynamics of tick-borne diseases. Through this emerging technology, we show the major role played by white-tailed deer in facilitating the emergence of *A. americanum*–associated zoonoses. However, the apparent contributions of various other hosts to pathogen transmission highlight the need for a community approach to understanding vector-borne zoonoses. Future applications of these methods will generate information for approaching a variety of topics of pressing concern to public health, including the potential impact of anthropogenic landscape change on human risk of exposure to zoonotic pathogens.

Acknowledgments

We thank Jonathan Chase, Felicia Keesing, Richard Ostfeld, the Chase Laboratory group, and 2 anonymous reviewers for helpful comments and suggestions. We also thank Monique Gaudreault-Keener for help with laboratory analyses and Russell Blaine, Kelly Oggenfuss, Richard Ostfeld, Maria Thaker, and Walter Wehtje for help with obtaining vertebrate tissue samples.

This research was supported by a Doctoral Dissertation Improvement Grant from the National Science Foundation, a Science to Achieve Results Graduate Fellowship from the Environmental Protection Agency, and a Lewis and Clark Fund for Exploration and Field Research Grant from the American Philosophical Society to B.F.A. and Washington University Research Support for Senior Administrators to R.E.T.

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Borrelia, *Ehrlichia*, and *Rickettsia* spp. in Ticks Removed from Persons, Texas, USA

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Data regarding the type, frequency, and distribution of tick-borne pathogens and bacterial agents are not widely available for many tick species that parasitize persons in the southern United States. We therefore analyzed the frequency and identity of pathogens and bacterial agents in ticks removed from humans and subsequently submitted to the Texas Department of State Health Services, Zoonosis Control Program, from October 1, 2004, through September 30, 2008. The data showed associations of bacterial agents and potential vectors. Tick-related illnesses may pose unidentified health risks in areas such as Texas, where incidence of human disease related to tick bites is low but well above zero and where ticks are not routinely suspected as the cause of disease. Cause, treatment, and prevention strategies can be better addressed through collecting sufficient data to establish baseline assessments of risk.

Data concerning the full distribution of tick-borne agents and their potential relationship to both emerging and characterized illnesses in the southern United States are not widely available. Persons who become ill after a tick bite may be at increased risk because a tick bite may not be considered as the source of the pathogen and because of the length of time that febrile illness may elude effective treatment. Detailed knowledge of the causative agents, their distribution, and their relationship to potential vectors is also lacking. Most tick survey data for microorganisms in the genera *Borrelia*, *Rickettsia*, and *Ehrlichia* have been collected in areas where the associated diseases are considered endemic. Lyme disease, Rocky Mountain spotted fever, or human monocytotrophic ehrlichiosis are not considered to be endemic to Texas. Studies of microorganisms carried in ticks in non–disease-endemic areas might provide information about potentially pathogenic organisms, their vectors, and reservoirs. These data might also provide an opportunity to examine the ecology of emerging zoonoses for which different ecologic determinants for disease transmission may be present.

In 2000, the 77th Texas Legislature Subcommittee on Administration prepared a report addressing the potentially severe nature of tick-borne disease in Texas. As of October 1, 2004, the Tick-Borne Disease Research Laboratory at the University of North Texas Health Science Center (UNTHSC) became the primary facility for testing ticks submitted to the Texas Department of State Health Services (TX DSHS).

Methods

From October 1, 2004, through September 30, 2008, tick specimens were submitted to UNTHSC through the Zoonosis Control Program of the TX DSHS. Only ticks that had been attached to a person were submitted to UNTHSC, where they were screened for the genera *Borrelia*, *Rickettsia*, and *Ehrlichia* with genus-specific PCRs. Ticks were identified to the species level by TX DSHS entomologists before being transferred to UNTHSC (*1*–*3*). Poor condition of some specimens made identification by morphologic examination difficult. Unidentified ticks were conclusively identified by molecular methods developed at UNTHSC, which used amplification of 12S rDNA (Table 1) and sequence determination (data not shown). Additionally, the identity of any tick containing an organism not previously reported in that species was also confirmed by the same molecular methods. Of all tick specimens, 10% were screened

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DOI: 10.3201/eid1603.091333

by the same molecular identification technique to verify the accuracy of morphologic identification.

Ticks were bisected laterally by using aseptic technique and a sterile scalpel blade. For independent verification of results, half of each tick was stored in 100% ethanol at –80°C. For larvae and nymphs, the entire tick was used for DNA extraction. Total DNA was isolated from the second half by using an E.N.Z.A. Mollusc DNA Isolation Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to manufacturer's recommended protocol. Extracted DNA was subjected to PCR that used primers for the amplification of the tick's 12S rDNA or *Borrelia* spp., *Ehrlichia* spp., and *Rickettsia* spp. genes (Table 1).

The locations of PCR setup and PCR product handling were physically separated. Reaction setup was performed in a class II type B2 biological safety cabinet that had been cleaned with 0.6% sodium hypochlorite daily and UV irradiated for 30 min before and after each use. To minimize risk for contamination, pipettor sets were dedicated to specific functions, i.e., reagent dispensing, template isolation, PCR setup, and template handling. Certified DNA/RNase-free filter barrier tips were used to prevent aerosol contamination. PCR setup was never performed in the presence of isolation materials, and reagent handling was separated both physically and temporally from templates. PCR assays were performed in duplicate with appropriate controls.

A typical, initial PCR was performed in a 25-μL reaction volume by using 5 pmol/L of each appropriate primer in conjunction with a final reaction concentration of 1× GeneAmp PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 160 ng/μL bovine serum albumin, 1.0 mmol/L $MgCl_2$, 200 μmol/L of each dNTP, 1.25 U Amplitaq (Applied Biosystems), and 10 μL of template. To establish the species of the tick specimen, we amplified 12S rDNA with

Table 1. Nucleotide sequence of primers used for PCR screening of tick specimens removed from humans, Texas, October 1, 2004, to September 30, 2008*

Primer name	Gene	Primer sequence (5′ → 3′)	Specificity	Screen	T_M	Reference
Tick DNA						
85F	*12S*	TTAAGCTTTTCAGAGGAATTTGCTC	Unknown	Primary	54.0	This study
225R	*12S*	TTTWWGCTGCACCTTGACTTAA	Unknown	Primary	52.7	This study
Borrelia spp.						
FlaLL	*flaB*	ACATATTCAGATGCAGACAGAGGT	Genus	Primary	58.3	(*4*)
FlaRL	*flaB*	GCAATCATAGCCATTGCAGATTGT	Genus	Primary	58.9	(*4*)
FlaLS	*flaB*	AACAGCTGAAGAGCTTGGAATG	Genus	Primary	57.5	(*4*)
FlaRS	*flaB*	CTTTGATCACTTATCATTCTAATAGC	Genus	Primary	53.3	(*4*)
BL-Fla 522F	*flaB*	GGTACATATTCAGATGCAGACAGAGGG	*B. lonestari*	Primary	61.3	This study
BL-Fla 1182R	*flaB*	GCACTTGATTTGCTTGTGCAATCATAGCC	*B. lonestari*	Primary	64.0	This study
BL-Fla 662F	*flaB*	CTGAAGAGCTTGGAATGCAACCTGC	*B. lonestari*	Primary	62.8	This study
BL-Fla 860R	*flaB*	GAGCTAATCCCACCTTGAGCTGG	*B. lonestari*	Primary	61.2	This study
BL-Fla 341F	*flaB*	AGCTGATGATGCTGCTGGTATGGG	Genus	Alternate	63.2	This study
BL-Fla 730R	*flaB*	GCTTGTGCTCCAGTTAGTGATGCTGG	Genus	Alternate	64.1	This study
BL-16S 227F	*16S*	TCACACTGGAACTGAGATACGGTCC	Genus	Alternate	62.1	This study
BL-16S 920R	*16S*	GAATTAAACCACATGCTCCACCGC	Genus	Alternate	61.0	This study
BL-HSP 71F	*groEL*	CTTATGTTGAAGGAATGCAATTTGA	*B. lonestari*	Alternate	55.6	This study
BL-HSP 271R	*groEL*	CAATATCTTCAGCAATAATTAGCAAAGGT	*B. lonestari*	Alternate	58.2	This study
Rickettsia spp.						
Rr.190 70P	*rompA*	ATGGCGAATATTTCTCCAAAA	Genus	Primary	52.5	(*5*)
Rr.190 602N	*rompA*	AGTGCAGCATTCGCTCCCCCT	Genus	Primary	64.9	(*5*)
BG1–21	*rompB*	GGCAATTAATATCGCTGACGG	Genus	Alternate	55.6	(*6*)
BG2–20	*rompB*	GCATCTGCACTAGCACTTTC	Genus	Alternate	55.2	(*6*)
RrCS 372	*gltA*	TTTGTAGCTCTTCTCATCCTATGGC	Genus	Alternate	59.0	(*7*)
RrCS 989	*gltA*	CCCAAGTTCCTTTAATACTTCTTTGC	Genus	Alternate	57.5	(*7*)
Primer 1	*17kDa*	GCTCTTGCAACTTCTATGTT	Genus	Alternate	52.3	(*8*)
Primer 2	*17kDa*	CATTGTTCGTCAGGTTGGCG	Genus	Alternate	57.9	(*8*)
Ehrlichia spp.						
Ehr DSB 330F	*dsb*	GATGATGTCTGAAGATATGAAACAAAT	Genus	Primary	55.5	(*9*)
Ehr DSB 728R	*dsb*	CTGCTCGTCTATTTTACTTCTTAAAGT	Genus	Primary	56.6	(*9*)
ECC-F	*16S*	AGAACGAACGCTGGCGGCAAGCC	Genus	Alternate	68.1	(*10*)
ECB-R	*16S*	CGTATTACCGCGGCTGCTGGCA	Genus	Alternate	65.6	(*10*)
ECAN-F	*16S*	ATTTATAGCCTCTGGCTATAGGA	*E. canis*	Alternate	54.9	(*11*)
HE1-F	*16S*	CAATTGCTTATAACCTTTTGGTTATAAAT	*E. chaffeensis*	Alternate	55.6	(*12*)
EE72-F	*16S*	AATTCCTAAATAGTCTCTGACTATT	*E. ewingii*	Alternate	52.6	(*11*)
HE3-R	*16S*	TATAGGTACCGTCATTATCTTCCCTAT	Genus	Alternate	57.6	(*13*)

*T_M, melting temperature, °C.

the following cycle parameters: 95°C for 5 min; then 40 cycles each consisting of 95°C for 30 s, 45°C for 30 s, 72°C for 60 s; and a final 72°C extension for 5 min. Thermal cycling parameters for the initial PCRs of bacterial genes were 95°C for 5 min; then 40 cycles each consisting of 95°C for 60 s, 55°C for 60 s, 72°C for 30 s; and a final 72°C extension for 5 min. Nested PCR was performed by using the same reaction setup and 1.0 μL of amplified PCR mix as template. Nested PCR setup was performed in a dedicated dead air space cabinet that had been decontaminated in the same manner as the class II type B biosafety cabinet. The thermal cycling profile for the nested reactions was 95°C for 5 min; then 30 cycles each consisting of 95°C for 60 s, 55°C for 60 s, 72°C for 60 s; and a final 72°C extension for 5 min.

Verification of amplification was performed by agarose gel electrophoresis, followed by staining with 1X SYBR Green I (BioWhittaker Molecular Applications ApS, Rockland, ME, USA). Amplicons were examined with a UVP EC3 Imaging System (UVP, LLC, Upland, CA, USA) and subsequently analyzed by VisionworksLS Image Acquisition and Analysis Software (UVP, LLC). Secondary PCR systems (Table 1) were used to confirm positive results and did not contain primers that would amplify control DNA commonly used in the laboratory. Unincorporated primers were removed from samples producing amplicons before sequence determination by using ExoSAP-IT (USB Corporation, Cleveland, OH, USA).

DNA sequencing was performed for both strands of the PCR amplicons by using a BigDye Terminator Cycle Sequencing Kit, version 3.1 (Applied Biosystems). Unincorporated dye terminators were removed before electrophoresis by using Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD, USA). Capillary electrophoresis was performed by using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Final sequence analysis and editing was performed by using Sequencer 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). Using BLASTN, version 2.2.10 (www.ncbi.nlm.nih.gov/blast/Blast.cgi), we then compared edited sequence data with genetic sequences from characterized examples of *Borrelia* spp., *Rickettsia* spp., and *Ehrlichia* spp. published in GenBank.

Results

A total of 903 ticks, representing 11 tick species, were submitted to UNTHSC from 138 of 254 Texas counties. Of these, 144 ticks contained the DNA of at least 1 of the agents in the genera *Borrelia*, *Ehrlichia*, or *Rickettsia* (Table 2). The most common tick species submitted were *Amblyomma americanum,* followed by *Dermacentor variabilis.* Spotted fever group *Rickettsia* spp. (SFGR) were the most common bacteria detected. Genetic material from SFGR was identified in *A. americanum, A. cajennense*, *D. variabilis*, *Ixodes scapularis*, *and Rhipicephalus sanguineus* ticks. Of all tick species submitted, minimum SFGR infection rates (MIRs) were highest for *A. americanum* (20.98%) and *D. variabilis* (47.37%) ticks. The most predominant SFGR sequences amplified were identical to those of *Candidatus* Rickettsia amblyommii (AY062007). Some contained a single-nucleotide difference relative to AY062007 (data not shown). SFGR amplicons produced from *Ixodes* spp. ticks were identical to those of *I. scapularis* endosymbiont isolates (EU544296, EF689740, EF689737) and shared ≥99% identity with *Candidatus* Rickettsia cooleyi (AF031535) (*14*) or an uncharacterized rickettsial endosymbiont previously reported for *I. scapularis* (AB002268) ticks (*15*). Amplicons with a DNA sequence identical to that of *R. parkeri* strains (U43802) (*16*), (EF102238) (*17*), and (FJ986616) were produced by 4 *D. variabilis* and 1 *Rh. sanguineus* tick samples. Amplicons identical to *R. peacockii* (CP001227) were produced by 2 *A. americanum*, 2 *D. variabilis*, and 1 *I. scapularis* tick samples. Amplicons identical to *R. rhipicephali* (U43803) and at least 99% similar to other *R. rhipicephali* strains (EU109175, EU109177, EU109178) (*18*) were produced by 1 *Rh. sanguineus* tick sample.

DNA sequences consistent with those of *Borrelia* spp. were derived from *A. americanum, A. cajennense*, *D. variabilis*, and *I. scapularis* ticks. The most commonly encountered *Borrelia* genetic material demonstrated at least 99% sequence identity or was identical to that of previously sequenced *Candidatus* Borrelia lonestari isolates (AY850063, AF538852) (*19*). Additionally, a borreliae *flaB* sequence was generated from 1 *D. variabilis* tick, which had 94% sequence similarity with that of *Candidatus* Borrelia texasensis (AF264901) (*20*) and sequences amplified from an uncultured *Borrelia* sp. from the bat tick *Carios kelleyi* (EF688577, EF688579) (*21*) and (EU492387). The *flaB* sequence contained 11 single-nucleotide polymorphisms relative to the corresponding section of AF264901. The *Borrelia* sp. 16S rDNA sequence generated from the same *D. variabilis* tick was also identical to that published for *Candidatus* B. texasensis (AF467976) (*20*,*22*). This tick was submitted from Webb County, the same Texas county from which the borreliae that produced GenBank sequence AF264901 were isolated. A single *I. scapularis* specimen produced the *flaB* sequence, which had 99% identity with *B. burgdorferi* (AE000783) (*23*).

Genetic data consistent with those from *Ehrlichia* spp. were observed for *A. americanum*, *A. cajennense,* and *A. maculatum* ticks. Amplicons produced from *A. americanum* and *A. maculatum* ticks were 99% similar to the homologous region of the *E. chaffeensis* disulfide oxidoreductase gene (*dsb*) sequences in GenBank (CP000236) (*24*). A single sample from *A. cajennense* ticks produced a DNA sequence that was 97% similar to that of the CP000236 se-

quence and contained 8 single-nucleotide polymorphisms relative to the similar sequence. Several single-nucleotide polymorphisms locations are at the same position as nucleotide differences identified between the *dsb* gene of *E. ewingii* (AY428950) (*25*) and *E. canis* (AF403710) (*26*). The nucleotide polymorphisms found within the *dsb* gene did not change the predicted amino acid sequence in relation to *E. chaffeensis* (data not shown).

Discussion

By screening a diverse group of Texas tick species for a range of microorganisms and potential pathogens, we identified several novel associations: *Candidatus* B. lonestari in *A. cajennense* ticks, *E. chaffeensis* in *A. cajennense* ticks, and *A. maculatum*, and *R. parkeri* in *D. variabilis* ticks (Table 3). Because the geographic distribution of diseases caused by the agents is generally characterized by the distribution of the tick vectors, these findings provide insights regarding the distributions and endemicity of several potential emerging tick-borne agents.

SFGR were the most commonly observed agents in this survey. Both *Candidatus* R. amblyommii and *Candidatus* R. cooleyi are not well studied and are of undetermined pathogenicity. Current average SFGR seropositivity in Texas residents is also unknown, yet prior estimates indicate that it is higher than would be assumed from cases of Rocky Mountain spotted fever reported to the TX DSHS (*27*). Transmission through blood products has been noted previously (*28*,*29*). Unreported subclinical infections might cause concern about local blood products and could potentially compromise immunodeficient transfusion recipients. Additionally, detection of *R. amblyommii* in questing *A. americanum* larvae suggests transovarial transmission of the microbe, and the likelihood of pathogen transmission

Table 2. Number and identity of ticks submitted to University of North Texas Health Science Center by the Texas Department of State Health Services Zoonosis Control Program, October 1, 2004, to September 30, 2008*

	No. positive/no. tested									
	Borrelia spp.			*Ehrlichia* spp.			*Rickettsia* spp.			
Tick	UNE	PE	E	UNE	PE	E	UNE	PE	E	Total
Amblyomma americanum										
Adult male	0/0	1/116	0/1	0/0	0/116	0/1	0/0	25/116	0/1	26/117
Adult female	0/0	1/109	0/11	0/0	2/109	0/11	0/0	23/109	4/11	30/120
Nymph	0/0	1/92	1/27	0/0	0/92	0/27	0/0	18/92	9/27	29/119
Larva	0/0	0/11	0/0	0/0	0/11	0/0	0/0	0/11	0/0	0/11
A. cajennense										
Adult male	0/0	0/44	0/2	0/0	1/44	0/2	0/0	0/44	0/2	1/46
Adult female	0/0	1/56	0/3	0/0	0/56	0/3	0/0	0/56	0/3	1/59
Nymph	0/0	0/52	0/3	0/0	0/52	0/3	0/0	3/52	1/3	4/55
Larva	0/0	0/12	0/0	0/0	0/12	0/0	0/0	1/12	0/0	1/12
A. maculatum										
Adult male	0/0	0/7	0/0	0/0	1/7	0/0	0/0	0/7	0/0	1/7
Adult female	0/0	0/1	0/1	0/0	0/1	0/1	0/0	0/1	0/1	0/2
Nymph	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/1	0/1	0/1
Larva	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0
Dermacentor variabilis										
Adult male	0/1	0/71	0/1	0/1	0/71	0/1	0/1	4/71	0/0	4/73
Adult female	0/3	1/84	0/16	0/3	0/84	0/16	0/3	6/84	1/16	8/103
Nymph	0/0	0/0	0/2	0/0	0/1	0/2	0/0	0/0	0/2	0/2
Larva	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0
Ixodes scapularis										
Adult male	0/0	0/4	0/0	0/0	0/4	0/0	0/0	0/4	0/0	0/4
Adult female	0/0	0/41	0/22	0/0	0/41	0/22	0/0	26/41	6/22	32/63
Nymph	0/0	1/8	0/1	0/0	0/8	0/1	0/0	4/8	0/1	5/9
Larva	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Rhipicephalus sanguineus										
Adult male	0/0	0/23	0/0	0/0	0/23	0/0	0/0	0/23	0/0	0/23
Adult female	0/2	0/35	0/6	0/2	0/35	0/6	0/2	1/35	0/6	1/43
Nymph	0/0	0/5	0/15	0/0	0/5	0/15	0/0	0/5	1/15	1/20
Larva	0/0	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/1	0/1
Total	0/6	6/772	1/112	0/6	4/772	0/112	0/6	111/772	22/112	144/890

*Testing by PCR. Only tick species that showed evidence of containing *Borrelia*, *Ehrlichia*, or *Rickettsia* spp. are shown. Seven specimens of *Otobius megnini,* 2 of *Amblyomma inornatum* and *Dermacentor albipictus,* and 1 each of *Dermacentor andersonii* and *Dermacentor nigrolineatus* ticks were submitted during the project period. After clarification with the submitter of the *D. andersonii* specimen, it was concluded that the tick attachment may have occurred in Colorado. UNE, unengorged; PE, partially engorged; E, engorged.

Table 3. No. ticks containing bacterial DNA sequences, Texas, October 1, 2004, to September 30, 2008*

Tick species	Bacterial agent								
	Borrelia spp.	*B. burgdorferi*	*Candidatus* Borrelia lonestari	*Ehrlichia chaffeensis*	*Candidatus* Rickettsia amblyommii	*Candidatus* Rickettsia cooleyi	*Rickettsia parkeri*	*R. peacockii*	*R. rhipicephali*
Amblyomma americanum			4	2	77			2	
A. cajennense			1	1	5				
A. maculatum				1					
Dermacentor variabilis	1				4		4	2	1
Ixodes scapularis		1				35		1	
Rhipicephalus sanguineus					1		1		
Total	1	1	5	4	87	35	5	5	1

*Ticks submitted to the Texas Department of State Health Services and identified by the University of North Texas Health Science Center, Tick-Borne Disease Research Laboratory. Only those tick species that showed evidence of containing *Borrelia*, *Ehrlichia*, or *Rickettsia* spp. are shown.

by larvae could be magnified by their habit of mass attack (huge numbers on a single host).

An overall *Borrelia* spp. MIR of 1.1% was observed for the entire 4-year collection. Prevalence of *Candidatus* B. lonestari in ticks from Texas was low. However, *Candidatus* B. lonestari sequences were detected in *A. americanum* ticks regardless of geographic origin. The MIR was slightly higher for *A. americanum* (2.53%) ticks during periods when that tick was the most abundant species parasitizing humans (October 1, 2007 through October 1, 2008). These rates are within ranges previously established in the literature (*30*–*32*). A single isolate of *Candidatus* B. lonestari was observed in *A. cajennense* ticks. This represents the potential for *Candidatus* B. lonestari to use hard ticks of species other than *A. americanum* in its maintenance cycle and suggests that *Candidatus* B. lonestari may occur in areas outside the natural distribution of *A. americanum* ticks. An MIR of 1.3% for *Borrelia* spp. was found for in *D. variabilis* and may indicate the presence of uncharacterized borreliae strains in Texas tick populations.

A. cajennense ticks have been associated with *E. ruminantium* (*33*) and spotted fever group *Rickettsia* spp. (*34*). According to seropositivity in a human population in Argentina, these ticks have also been suspected of transmitting ehrlichiosis (*35*). The presence of *E. chaffeensis* in an *A. cajennense* tick seems novel. Long et al. (*13*) suggest an *E. ewingii* MIR of 7.6% in southcentral Texas *A. americanum* ticks. Similar results for *Ehrlichia* spp. in *A. cajennense* tick populations may be plausible.

Screening ticks for a range of bacterial agents has provided several additional associations. These findings provide insights regarding the distributions and endemicity of potentially pathogenic and emerging tick-borne agents. Some of these tick-borne agents may pose an unknown health risk. Because of the wide distribution of these ticks, accurate assessments of the frequency of bacterial agents in these tick populations, their potential for causing human disease, and the ability for these tick species to act as competent vectors are warranted. Continued study and monitoring will play a vital role in public health assessment for related disease risks.

Acknowledgments

We thank Chris Paddock and Bruce Budowle for their review of the manuscript.

This project was supported by the State of Texas.

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New Endemic *Legionella pneumophila* Serogroup I Clones, Ontario, Canada

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The water-borne pathogen *Legionella pneumophila* serogroup 1 (Lp1) is the most commonly reported etiologic agent of legionellosis. To examine the genetic diversity, the long-term epidemiology, and the molecular evolution of Lp1 clinical isolates, we conducted sequence-based typing on a collection of clinical isolates representing 3 decades of culture-confirmed legionellosis in Ontario, Canada. Analysis showed that the population of Lp1 in Ontario is highly diverse and combines lineages identified worldwide with local strains. Identical types were identified in sporadic and outbreak-associated strains. In the past 15 years, the incidence of some lineages distributed worldwide has tended to decrease, and local endemic clones and lineages have emerged. Comparative geographic distribution analysis suggests that some lineages are specific to eastern North America. These findings have general clinical implications for the study of Lp1 molecular evolution and for the identification of Lp1 circulating strains in North America.

Legionella species are implicated in 2 clinical syndromes: Legionnaires' disease (LD) and Pontiac fever, which are collectively known as legionellosis. Pontiac fever is a self-limited, influenza-like illness, whereas Legionnaires' disease is a common cause of serious bacterial pneumonia (*1*,*2*).

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DOI: 10.3201/eid1603.081689

Among the 52 species and 70 serogroups of *Legionella* species (*3*), *L. pneumophila* is the major cause of sporadic and outbreak legionellosis (91.5%), and serogroup 1 is the predominant serotype (84.2%) (*4*). In industrialized countries, *L. pneumophila* is the second most common pathogen detected in cases of community-acquired pneumonia that requires patient admission to intensive care units (*5*,*6*).

During an outbreak or after the detection of sporadic cases, appropriate identification and typing methods are essential for epidemiologic investigations. Adequate typing methods are also crucial to determine the degree of relatedness of bacteria and to enable the reconstruction of microevolutionary events (*7*). On the basis of analysis of 7 loci, a standard sequence-based method for the typing of *L. pneumophila* serogroup 1 (Lp1) was developed by the European Working Group for *Legionella* Infections (EWGLI) (*8*,*9*).

In previous population-based studies, a *Legionella* sequence-based typing (SBT) scheme was used to analyze clinical strains either from Europe or with limited time-span coverage (*10*–*12*). In the present study, we applied SBT to examine the genetic diversity, the long-term epidemiology, and the molecular evolution of Lp1 clinical isolates using a population-based collection that encompassed isolates from 30 years of culture-confirmed legionellosis cases in Ontario.

Methods

Source of Isolates

Legionellosis is a notifiable disease in Ontario (population 13 million persons). Since 1978, the diagnosis of *Legionella* infections has been centralized at the Ontario Public Health Laboratory (OPHL). This laboratory serves as the *Legionella* reference laboratory and performs all testing for outbreak investigations and most testing of clin-

ical specimens. Therefore, isolates analyzed in this study are representative of the strains isolated in Ontario in the past 3 decades. Information available in the Ontario database includes dates of onset of illness, patient's age and sex, and city and hospital or healthcare facility from which specimens were submitted (*13*). No specimens were submitted for *Legionella* isolation from 1978 through 1979. From 1980 through 1985, a mean ± SD of 424.1 ± 281.3 specimens was submitted for isolation every year. From 1986 through 2007, a mean ± SD of 1,783.5 ± 258.4 specimens was submitted for isolation every year. The mean ± SD number of Lp1 culture-confirmed cases/year during the study period was 7.4 ± 3.5. The proportion of culture-confirmed case-patients with *L. pneumophila* infection remained stable during the period of analysis, and 66% of the isolates were Lp1 (*13*).

Lung tissues, bronchial-alveolar lavage specimens, or sputum specimens were homogenized by using a tissue grinder, streaked on buffered charcoal yeast extract agar plates and incubated at 37°C (3–7 days). Species and serogroups were confirmed by direct immunofluorescent antibody assay and slide-agglutination (*14*,*15*). Isolates (n = 217) were stored at –80°C in trypticase soy broth supplemented with 5% horse blood. Twenty-three isolates obtained in 1996 and 1997 could not be used and were omitted from our analysis. Outbreaks were defined as ≥2 cases that were submitted from the same hospital or healthcare facility or with links to a common source with onset during the same 30-day period.

Sequence-based Typing

SBT using loci *fla*A, *pil*E, *asd*, *mip*, *mompS*, *proA,* and *neuA* was conducted according to the EWGLI scheme (*8*,*9*). Automated contig-assembly and base-calling of DNA sequence traces were performed by using the EWGLI sequence quality tool (*16*). The sequences obtained from this work are available in the EWGLI-SBT database (www.ewgli.org)

Phylogenetic and Allelic Diversity Analyses

Multiple sequence alignments of concatenated DNA sequences and phylogram construction were carried out with ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) by using the neighbor-joining method with 1,000 bootstrap replicates (*17*). Clonal analyses were performed by using eBURST3 (http://eBURST.mlst.net) with a group definition set to 6 identical alleles and sequence type (ST) allelic profiles were clustered with the unweighted pair group method with arithmetic mean (UPGMA) algorithm by using splits Tree4 (*18*). The standardized index of association (I_A^S) and the mean genetic diversity were calculated with LIAN 3.5 (*19*).

Ontario Health Regions and Rates Calculations

The 36 public health units of Ontario were aggregated into 7 health regions (OHRs) with populations ranging from ≈0.5 to 2 million persons: Toronto, South West, Central South, Central West, Central East, East, and North. Average rates were calculated by dividing disease counts by the Statistics Canada population estimates (*20*,*21*). OHR population estimates were not available before 1995 and were estimated from 1990 through 1995 and 2006 through 2007 by linear extrapolation.

Statistical Analyses and Mapping

Statistical analyses were performed by using STATA (StataCorp, College Station, TX, USA). Thematic maps were created by using ArcGIS (ESRI, Redlands, CA, USA).

Results

Lp1 Sequence-based Typing

The 194 isolates, collected from 1978 through 2007, were resolved into 62 STs (online Appendix Table, www.cdc.gov/EID/content/16/3/447-appT.htm). Seven STs were represented by at least 10 isolates, 13 STs consisted of groups containing 2–4 isolates, and 42 STs were represented by 1 isolate. In comparison to the EWGLI dataset, 42 STs have only been reported in North America, and 41 STs are unique to the province of Ontario. The ST with the largest number of isolates was ST1 (n = 31). STs previously reported in the EWGLI database and responsible for >9 cases in Ontario include ST36 (n = 10), ST37 (n = 21), ST42 (n = 10), and ST62 (n = 16). Two STs, specific to the province of Ontario, were detected in >9 legionellosis cases: ST211 (n = 15) and ST222 (n = 13). STs resolved from outbreak isolates were confirmed to be epidemiologically concordant since related isolates were assigned identical STs. ST211 strains were obtained from patients in 1 outbreak (n = 2) in 1993 and from 13 patients with sporadic cases. Seven of the 13 ST222 isolates were recovered from a legionellosis outbreak at a long-term care facility in Ontario in 2005 (*10*). ST226 was also differentiated from strains responsible for a suspected outbreak (n = 2) and is specific to Ontario.

Across the 7 loci, 99 alleles were identified. Three new alleles were found, 2 of which (*asd* 32 and *proA* 33) were identified in a ST357 strain isolated in 2002 from a patient with a sporadic case. The third new allele (*mompS* 52) was identified in a ST358 strain isolated in the South West OHR. At the individual loci level, the total number of alleles ranged from 10 at *flaA* to 21 at *mompS*. Because the population of Lp1 clinical isolates found in Ontario appeared to be distinct from the isolates reported in the EWGLI database, we performed linkage analyses and looked at

genetic diversity of our dataset compared to that of the EWGLI dataset. Linkage analyses showed that the I_A^S for the complete dataset was 0.4913. This value is comparable with the I_A^S of 0.494 in previous studies and suggests linkage disequilibrium in the dataset obtained from the population of clinical Lp1 isolates in Ontario (*22*). With a value of 0.8041 ± 0.0155, the mean ± SD genetic diversity of our dataset was similar to the mean genetic diversity of the EWGLI dataset.

Phylogenetic Analysis

The population structure of Lp1 clinical isolates from Ontario was analyzed by using 62 concatenated sequences of 7 loci and compared with results of cluster analysis deduced from SBT allelic profiles. Three major clusters were visually identified from the phylogenetic tree (Figure 1). All clusters contain isolates from outbreak and sporadic cases. None of the identified clusters or subgroups were exclusively formed with strains identified with STs specifically reported in Ontario. This suggests that Ontario strains are phylogenetically related to strains found in the EWGLI dataset. Phylogenetic cluster I (n = 39) included the epidemic strain ST1 and 7 STs of sporadic cases. With 114 isolates and 46 STs, cluster II was the largest and most diverse group from the dataset. In this cluster, Ontario outbreak strains ST37 and ST211 were subgrouped with ST36 (Philadelphia strain). Cluster III comprised 8 STs and 41 isolates. With the exception of ST222 (n = 13), none of the STs grouped in this cluster were reported to be outbreak strains.

Next, the UPGMA algorithm was used to construct a dendrogram based on a matrix of pairwise allelic differences between the 62 STs of our dataset (Figure 2). The topology of the UPGMA dendrogram was partially congruent with the neighbor-joining tree based on allelic sequences. The UPGMA dendrogram contains 3 major clusters of related STs arbitrarily named A, B, and C (Figure 2). Cluster B contains all isolates of cluster II except ST210 and ST199, which grouped with cluster C. In contrast, STs found in clusters I and III were separated into clusters A and C. ST1 and ST52 clustered in a separate branch at the base of the dendrogram, (Figure 2), which suggests that they could be phylogenetically distant from other STs. However, this divergence was not observed with the neighbor-joining method. Based on this finding, for the rest of the analysis, we considered cluster II as a well-defined phylogenetic group and clusters I/III were analyzed as a single group.

Identification of Clonal Lineages

The eBURST clonal analysis of our strains showed that the province of Ontario presents a semiclonal population with 27 single isolates and 11 clonal groups (CGs) (Figure 2). With 54 isolates and 10 STs, CG1 was the clonal group

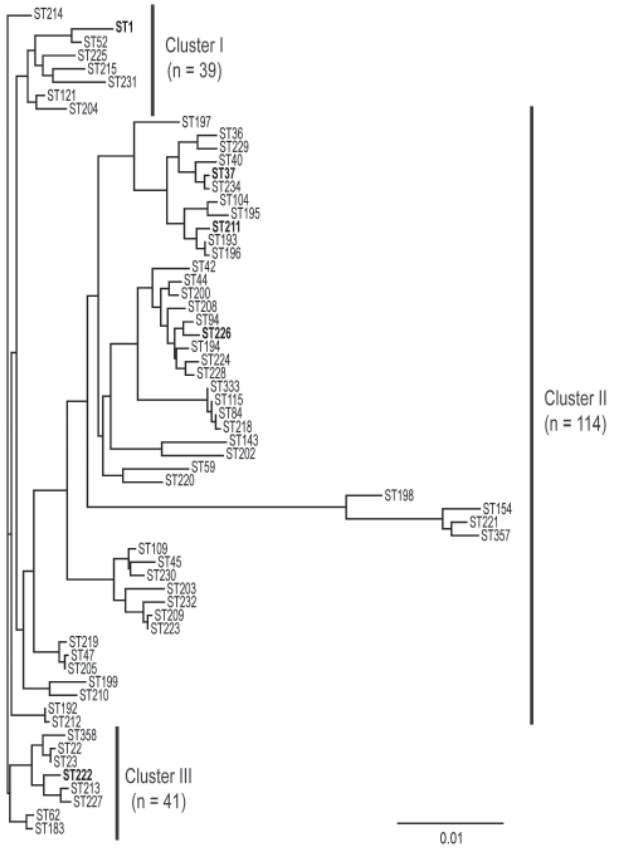

Figure 1. Phylogenetic analysis of *fla*A, *pil*E, *asd*, *mip*, *mompS*, *proA,* and *neuA* concatenated sequences from the 62 *Legionella pneumophila* serogroup 1 sequence types (STs) identified in Ontario. The tree was constructed with ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and the neighbor-joining method with 1,000 bootstrap replicates. Scale bar indicates genetic distances between sequences. STs in **boldface** were detected in outbreaks.

with the largest number of isolates and STs. This clonal group (27.8% of Ontario isolates) contained STs that were reported elsewhere (ST36, ST37, and ST104) but also STs that were unique to Ontario (ST193, ST195, ST196, ST197, ST211, and ST229). The founder of CG1 was predicted to be ST36 (bootstrap confidence [BC] = 68%), and the predominant single locus variant of this group was ST37 (n = 21). Members of CG1 were recovered from both sporadic and outbreak cases. CG2 (n = 7) only contained isolates with STs that are unique to Ontario and the ancestor of this group was predicted to be ST209 (BC = 28%). All isolates of CG2 were obtained from sporadic cases. Other clonal groups unique to Ontario included CG4 (n = 17), CG6 (n = 4) and CG7 (n = 2). Each of the 5 remaining clonal groups contained only 2 STs with combinations of STs specific to Ontario or previously reported in the EWGLI database.

We next did an eBURST comparative analysis of the SBT dataset from Ontario with the EWGLI database.

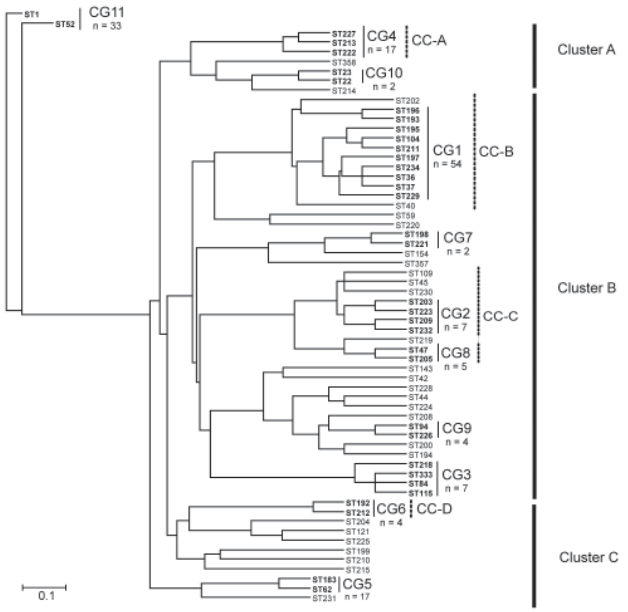

Figure 2. Dendrogram created by the unweighted pair group method with arithmetic mean method based on the 62 allelic profiles of 194 *Legionella pneumophila* serogroup 1 isolates. Clonal groups (CGs) identified by eBURST (http://eBURST.mlst.net) are indicated with solid lines, and STs included in CGs are in **boldface**. Ontario STs included in clonal complexes (CC) identified by comparative eBURST analysis with the European Working Group for *Legionella* Infections database are indicated with dashed lines. The 3 major clusters are indicated on the right of the figure with **bold** lines. The number of strains isolated in Ontario is indicated below CG. Scale bar indicates linkage distances.

STs detected in Ontario were only clustered in 17 of the 44 clonal complexes (CC) identified with the EWGLI database. This suggests that >60% of the clonal groups of the EWGLI dataset are absent from Ontario. Comparative eBURST analysis showed that CG1 is part of CC-B, which is the most diversified clonal complex in the international database (59 STs) (Figure 3). In addition to STs included in CG1, Ontario isolates ST40 and ST202 grouped in CC-B. Philadelphia strain ST36 was predicted to be the founder (BC = 91%) of this clonal complex. The high number of STs clustered in CC-B (12 STs) suggests that strains belonging to this group are evolving in Ontario (Figure 2).

Isolates from CG11 grouped within a CC comprising the highest number of EWGLI isolates (n = 490). The predicted founder of this clonal complex is ST1 and despite its high number of isolates, it comprises only 35 STs. Similarly, Ontario CG11 (n = 33) contained only 2 STs which suggests that strains belonging to these clonal groups have limited genetic variability.

Isolates from CG4 (n = 17) grouped with 2 North American clinical strains ST276 and ST289 in CC-A (Figure 3). Another clonal complex of interest was CC-C accounting for 16 STs (Figure 3). This complex included isolates grouped in CG2 and CG8 as well as 7 STs that were not identified in Ontario. Single isolates ST45, ST109, and ST230 from the Ontario dataset also grouped in CC-C. Finally, CC-D of the comparative eBURST analysis was identical to CG6 (Figure 2).

Temporal Trends of Lp1 Culture-confirmed Legionellosis in Ontario

During 1978–2007, differences could be observed in the distribution of specific STs, clonal complexes and phylogroups. During 1981–1994, ST1 strains were regularly isolated (n = 29) with case numbers ranging from 0 to 5 (peaking in 1983) (Figure 4, panel A). During this time period, ST1 caused 3 outbreaks and a significant increase in ST1 occurrence was observed (incidence rate ratio [IRR] 15.37, 95% confidence interval [CI] 3.67–64.43, p<0.001). In contrast, after 1994, prevalence of ST1 isolates decreased markedly (IRR 0.07, CI 0.02–0.27, p<0.001). On average, from 1978 through 2007, we observed a significant decrease of 9% per year of ST1 strains in Ontario (p<0.001). From 1995 through 2007, only 2 legionellosis ST1 culture-confirmed cases were reported.

In contrast, other STs have emerged in the past 20 years. ST47 was detected 3 times during 2003–2006. This

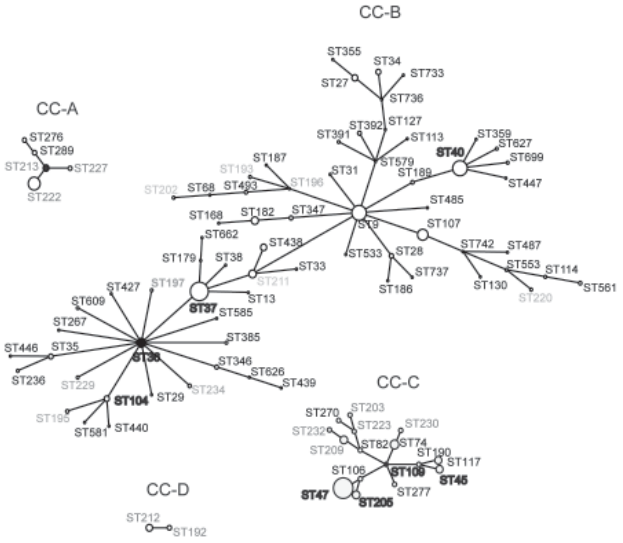

Figure 3. Representation of *Legionella pneumophila* serogroup 1 clonal complexes (CC) A, B, C, D obtained by comparative eBURST (http://eBURST.mlst.net) analysis between the Ontario collection and the European Working Group for *Legionella* Infections database. Each circle represents a single sequence type (ST). Size of the circle is proportional to the number of isolates. Dark circles represent predicted founder of each CC. Labels in **boldface** indicate STs found in both datasets, regular black characters indicate STs absent from the Ontario collection, light gray characters indicate STs exclusively found in Ontario. Solid lines represent single-locus variants.

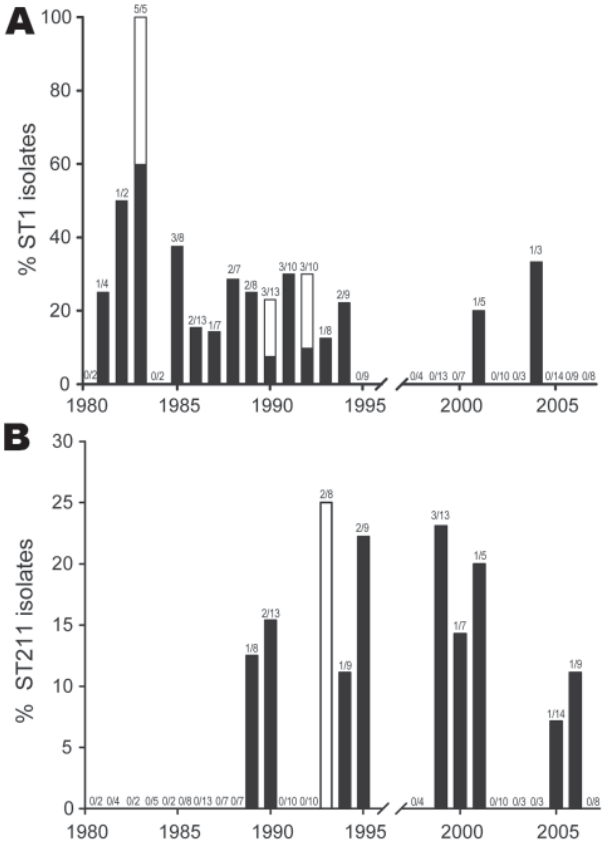

Figure 4. Prevalence of *Legionella pneumophila* serogroup 1 sequence type 1 (ST1) (A) and ST211 (B) endemic strains in Ontario. Black bar sections indicate proportion of strains from isolated cases and white bar sections indicate proportion of isolates from outbreaks.

strain was not isolated before 2003 in Ontario and it corresponds to the ST of the Lorraine strain. This emerging strain is highly prevalent in France where it was reported as the cause of 2 major outbreaks (*11*). Two other emerging strains that are unique to Ontario are ST211 and ST222 (Figure 4, panel B). ST211 was first isolated in 1989 accounting for 12.5% of clinical isolates. It was regularly isolated from 1990 through 2006, and sporadic cases peaked in 1999 (23.1%). ST222 was first reported in 1999, and the prevalence of this strain has significantly increased (IRR 1.30 per year, CI 1.12–1.53, p<0.001). Excluding outbreak isolates, ST222 accounted for 11.1% to 15.4% of clinical isolates in 1999, 2000, 2006, and 2007.

At the clonal complex level, some groups of strains have recently emerged in the province of Ontario. Ontario strains of CC-A were not detected in Ontario before 1992 and oscillated from 10% to 25% from 1998 through 2007 (Figure 5, panel A). This observation is consistent with the emergence of ST222, which is a major contributor of this clonal complex. Chronological evolution of Ontario isolates belonging to CC-C is also noteworthy because it appears to be an emerging clonal complex (Figure 5, panel B). From 1985 through 1988, the maximum incidence of isolates from this clonal complex was 15.4%. CC-C isolates were not reported in 1989 and 1995, but the incidence of CC-C isolates gradually increased from 1998 until 2007 (when it peaked at 37.5%).

Geographic Distribution of Lp1 Clinical Strains Isolated in Ontario

The geographic distribution of some individual STs, clonal complexes and phylogenetic clusters was not homogenous. With geographic ratios between 37.5% and 100%, the 7 STs that caused >10 clinical cases were all prevalent in the Toronto OHR. ST1 was widely distributed: South West, Central South, Central East, East, and Toronto OHRs. ST37 was found in all OHRs except the

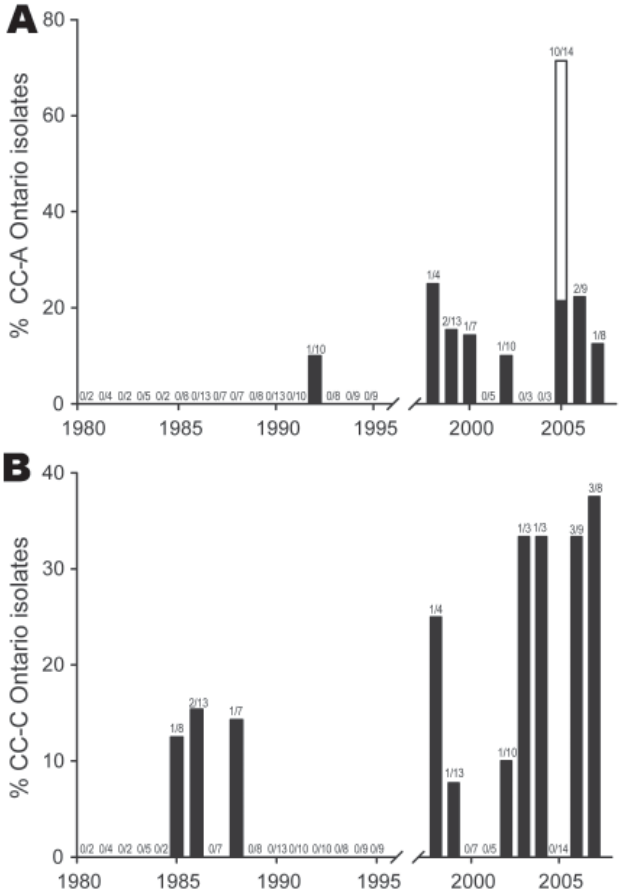

Figure 5. Incidence of Ontario *Legionella pneumophila* serogroup 1 isolates from clonal complexes (CC) A and C. CC-A (A) and CC-C (B) were identified by eBURST (http://eBURST.mlst.net) comparative analysis using the Ontario and the European Working Group for *Legionella* Infections international databases. Black bar sections indicate proportion of strains isolated during sporadic cases. White bar sections indicate proportion of outbreak isolates.

East OHR. ST62 was also homogeneously distributed in all OHRs with the exception of the North OHR. In contrast, distributions of ST36 and ST42 were not homogenous because they were only identified in the Central South, Central West, East, and Toronto OHRs. Distribution of ST211 was limited to the Toronto OHR. From 1978 through 2006, ST211 was identified 8 times in the same hospital. Excluding suspected linked cases, ST211 was exclusively detected 7 times in the Toronto OHR. Despite their recent emergence in 1999, ST222 strains were reported in multiple OHRs, including South West, Central West, Toronto, and East.

Comparative eBURST analysis between Ontario and EWGLI datasets grouped Ontario single isolates ST208 (n = 2) with ST257. ST257 was identified in a clinical case of legionellosis from New Hampshire, USA. This small clonal complex may be geographically restricted to eastern North America. Similarly, geographic distribution of CC-A (CG4, ST276, and ST289) was restricted to eastern North America. ST276 and ST289 were only reported in the states of New York and Connecticut (*23*). With the exception of the North OHR, strains belonging to CC-B were reported in all OHRs, although a high prevalence of CC-B isolates were identified in the Toronto OHR (63.2%). Without significant geographic prevalence, CC-C isolates were identified in all OHRs. CC-D comprised STs only reported in Ontario (Figure 3).

Geographic distributions of Ontario major phylogenetic groups were analyzed by mapping average rates of culture-confirmed Lp1 cases according to OHRs from 1990 through 2007 (Figure 6). Rates of clusters based on sequence-based types and phylogroups appeared to be partially dependent on geographic location. As expected from our distribution analyses of individual STs and clonal complexes, the Toronto OHR was more likely to have legionellosis cases caused by cluster I/III (0.062/100,000 persons/year) and cluster II (0.08/100,000 persons/year) than all other OHRs. With rates ranging from 0.02/100,000 person years for the North OHR to 0.08/100,000 person-years for the Toronto OHR, isolates from cluster II were unevenly reported in all OHRs. In contrast, legionellosis caused by cluster I/III were not identified in the North OHR and the rate for the Central OHR was only 0.01/100,000 person-years, which is 3.3× less than the rate reported for cluster II.

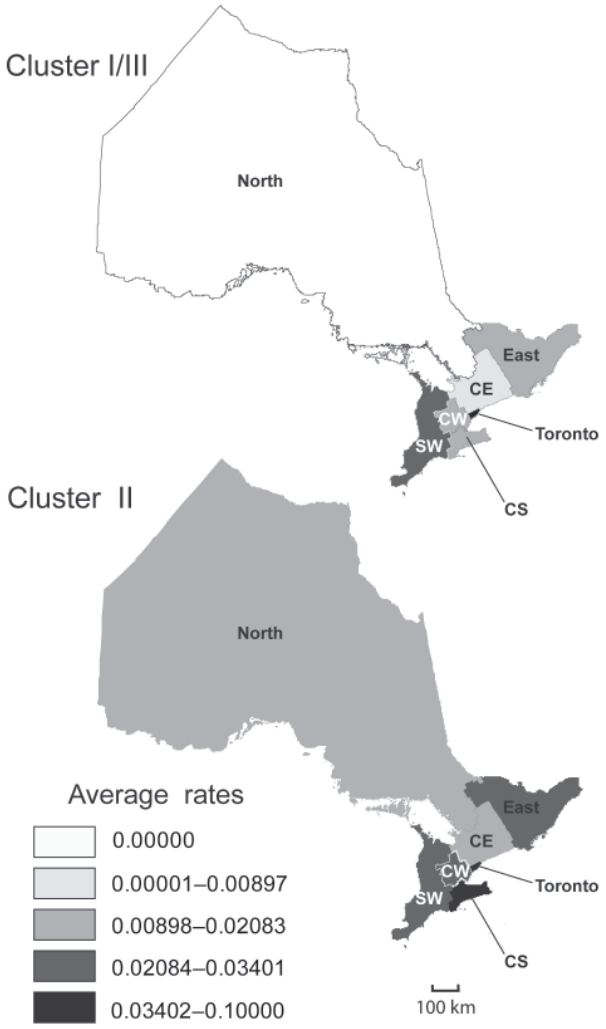

Figure 6. Geographic distribution of phylogenetic clusters II and I/III from 1990 through 2007. Rates are cases of infection with *Legionella pneumophilia* serogroup 1 clones per 100,000 persons per year. The province of Ontario was divided into 7 health regions (OHRs) with populations ranging from ≈0.5 to 2 million persons: Toronto, South West (SW), Central South (CS), Central West (CW), Central East (CE), East, and North.

Discussion

This report represents the first large-scale population-based SBT analysis of Lp1 clinical isolates within North America over a 30-year period. Sixty-two STs were identified among the isolates of the Ontario collection, which reflects a high degree of genetic diversity of Lp1 clinical isolates. Forty-one STs were unique to Ontario. Thus, the population of clinical Lp1 of this province consists of a combination of widely distributed and local isolates.

Although most sporadic cases were caused by isolates with a unique ST, some STs, like ST1, were found to be responsible for sporadic cases and outbreaks cases. This finding is in agreement with the recent identification of the Paris strain in sporadic and outbreak cases (*24*). Moreover, we have identified additional clones, specifically ST37, ST211, ST222, and ST226, which were also detected in sporadic and outbreaks cases. Although some small clonal groups such as CG2 exclusively comprised sporadic cases, our comparative analysis found no specific correlation be-

tween clonal complexes or phylogenetic clusters and ability to cause sporadic or outbreak cases.

A notable finding of our study is the high proportion of ST1 isolates identified, which supports the hypothesis that some specific Lp1 clones have gained widespread dissemination. This hypothesis was proposed after analyses of protein polymorphism and pulsed-field gel electrophoresis showed similar patterns from isolates distributed worldwide (*25*,*26*). Recently, the Paris strain was suggested to be one of these worldwide distributed strains because it has been identified in many European countries in patients and environmental samples (*24*,*27*). In France, the Paris strain was identified in 12.2% of culture-confirmed cases from 1998 through 2002, and it was shown to be the most prevalent endemic strain (*28*). Our study suggests that the notion of worldwide distributed strains could be broadened to include other sequence types such as ST36 or ST37. These 2 STs have been reported in European countries for clinical and environmental isolates and comprised 16% of STs from culture-confirmed cases in Ontario.

In previous studies, the clinical predominance and large distribution of ST1 suggested that it is a stable clone, well adapted to environmental survival or to host infection (*28*,*29*). Surprisingly, although ST1 was identified in 16.5% of the culture-confirmed cases of legionellosis over the past 30 years, our study also shows that the incidence of ST1 strains has decreased dramatically during the past 12 years. Because clonal analysis suggests that ST1 presents a limited genetic variability in our geographic area, we can hypothesize that its ability to colonize the environment or to be isolated by culture or its virulence might have been impaired in the recent years. In contrast, endemic clonal groups and clinical strains like ST211 have emerged in our geographic area in the past 15 years. A surveillance study recently reported a new endemic Lorraine strain (ST47) emerged in France (*11*). ST47 was only recovered 3 times over 30 years in Ontario, but CC-C, comprising ST47, is an emerging clonal complex in Ontario. Our analysis suggests that, globally, ST1 strains are being replaced by other emerging strains or clonal complexes.

Geographic distribution analysis of culture-confirmed population rates suggests that strains from cluster II are largely distributed in Ontario, whereas clusters I/III were mostly reported in the OHRs in close proximity to Lake Ontario. This finding could reflect differences in ecologic niches (either combined with degree of adaption of organisms to cause human disease or not). Some endemic emerging STs and clonal groups are exclusively detected in Ontario, in eastern North America, or in both. In the United States, the census regions with highest incidence rates for legionellosis are East North central and Middle Atlantic, at the proximity of the Great Lakes (*30*). *Legionella* species are abundant in surface waters and the Great Lakes ecosystem might represent an ideal ecologic niche for these bacteria. This hypothesis is in agreement with the identification of clonal complexes comprising isolates exclusively originating from eastern North America. This finding contrasts with findings of a recent population structure analysis of *L. pneumophila* that used allelic profiles from the EWGLI database that could not identify eBURST groups containing profiles originating from a single geographic area (*22*).

In conclusion, we showed that the population of clinical Lp1 in the province of Ontario is a combination of worldwide distributed and local strains. Our population of isolates might represent more severe cases as human respiratory samples are more frequently taken from patients requiring hospitalization, but the decreased prevalence of some clones and the emergence of local group of isolates suggest that the population of Lp1 has evolved or adapted to its environment during the past 30 years. Further research is required to explain the changing incidence of these STs and to investigate the fitness of emerging strains or clonal groups. Outcomes of this research will be helpful to improve surveillance programs for legionellosis as well as to ensure adequacy of clinical testing procedures with circulating strains.

Acknowledgments

We thank the European Working Group for *Legionella* Infections for assistance and for authorizing access to EWGLI database, and David J Farrell for reviewing the manuscript.

This study was supported by funding from the innovation program of Ontario Ministry of Government Services (no. 07007) and the Ontario Ministry of Health and Long term Care.

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Invasive *Haemophilus influenzae* Disease, Europe, 1996–2006

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An international collaboration was established in 1996 to monitor the impact of routine *Haemophilus influenzae* type b (Hib) vaccination on invasive *H. influenzae* disease; 14 countries routinely serotype all clinical isolates. Of the 10,081 invasive *H. influenzae* infections reported during 1996–2006, 4,466 (44%, incidence 0.28 infections/100,000 population) were due to noncapsulated *H. influenzae* (ncHi); 2,836 (28%, 0.15/100,000), to Hib; and 690 (7%, 0.036/100,000), to non–b encapsulated *H. influenzae*. Invasive ncHi infections occurred in older persons more often than Hib (median age 58 years vs. 5 years, p<0.0001) and were associated with higher case-fatality ratios (12% vs. 4%, p<0.0001), particularly in infants (17% vs. 3%, p<0.0001). Among non-b encapsulated *H. influenzae*, types f (72%) and e (21%) were responsible for almost all cases; the overall case-fatality rate was 9%. Thus, the incidence of invasive non–type b *H. influenzae* is now higher than that of Hib and is associated with higher case fatality.

Haemophilus influenzae is differentiated according to its capsular polysaccharide composition into 6 serotypes (a–f) and noncapsulated strains (*1*). Before routine vaccination, *H. influenzae* type b (Hib) caused >80% of invasive *H. influenzae* infections, primarily in healthy children <5 years of age (*2*). In contrast, non–type b *H. influenzae* usually causes opportunistic infections (*3*–*7*), particularly among elderly persons, who often have predisposing medical conditions such as chronic respiratory disease or immunosuppression (*6*–*11*).

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DOI: 10.3201/eid1603.090290

The introduction of the Hib conjugate vaccine into national childhood immunization programs in the 1990s has resulted in a marked and sustained reduction in the incidence of invasive Hib disease in many countries (*2*). However, concern exists about the long-term effectiveness of the Hib immunization programs (*12*) and possible disease replacement by other *H. influenzae* strains (*13*). Because Hib conjugate vaccine reduces pharyngeal carriage (*14*), other *H. influenzae* strains theoretically could take its place and cause invasive disease (*13*,*15*). Elimination of Hib carriage also may reduce natural boosting of immunity, thereby resulting in lower Hib antibody and increased susceptibility to invasive Hib disease in the long term (*16*).

In 1996, a collaborative surveillance network was established in Europe to describe the impact of routine Hib vaccination on the epidemiology of invasive *H. influenzae* disease. By 2006, a total of 28 countries participated in surveillance, and 14 countries, comprising an annual denominator population of 150 million persons, routinely serotyped all invasive clinical *H. influenzae* isolates. We describe the epidemiology of invasive *H. influenzae* disease in countries with established national Hib immunization programs, devoting particular attention to invasive non–type b *H. influenzae* disease.

Methods

A 3-year European Union–funded study (the BIOMED II Hib surveillance project) was initiated in 1996 to study the epidemiology of invasive *H. influenzae* after the introduction of the Hib conjugate vaccine into national infant immunization programs. In 1998, this program was renamed European Union Invasive Bacterial Infection Surveillance (EU-IBIS) and expanded to include more countries; by

[1]Participants listed at the end of this article.

2006, a total of 28 countries routinely reported cases to EU-IBIS (www.euibis.org). Participating countries reported cases of invasive *H. influenzae* disease to a central database, with basic demographic details, clinical syndrome, outcome, specimen site, and serotyping data on isolates. The UK Health Protection Agency *Haemophilus* Reference Unit (HRU) coordinated development of standardized laboratory protocols for growing, serotyping, and PCR genotyping *H. influenzae* and exchange of clinical isolates to ensure consistency of results. HRU also provided genotypic confirmation of serotypes for countries without established reference facilities and regularly distributed quality assurance strains to participating laboratories to ensure comparability of results. Annual reports were made available to participants at meetings and on the EU-IBIS website.

Invasive *H. influenzae* disease was defined as isolation of the organism from a normally sterile site. A case of meningitis was defined as *H. influenzae* cultured from cerebrospinal fluid or clinical and/or radiologic features of meningitis with blood culture positive for *H. influenzae*. Other clinical presentations, including epiglottitis, pneumonia, cellulitis, and osteomyelitis, were defined as isolation of *H. influenzae* from a normally sterile site (usually blood cultures but occasionally from another sterile site, e.g., joint fluid in septic arthritis or pleural fluid in empyema) in a person with clinical signs and symptoms consistent with that presentation. Bacteremia was defined as growth of *H. influenzae* from blood cultures only, with no distinctive clinical syndrome identified.

This study included only countries that routinely vaccinated children against Hib before 2000 and serotyped at least 50% of all clinical isolates. Detailed surveillance methods for all participating countries are available at www.euibis.org. Germany and Israel reported data for children only and were included in some of the analyses. Within the United Kingdom, data from England and Wales were collected separately from Scotland because the surveillance program in England and Wales is separate from that in Scotland. In Greece, during 1996–2002, surveillance was limited to a single prefecture, Attiki, and provided data only for persons <15 years of age; after 2002, national data were available for Greece. Italy initially relied solely on laboratory reporting with voluntary notification of confirmed cases of meningitis, but a more active laboratory-based surveillance system was established in 8 Italian regions in 1997–1998 (Campania, Liguria, Lombardia, Piemonte, Puglia, Toscana, Trento, and Veneto), 7 regions in 1999–2002 (Lombardia was no longer included), and nationally thereafter. All data were collected as part of enhanced national surveillance and rendered anonymous at the source.

Data were entered by using Microsoft Excel (Microsoft, Redmond, WA, USA), and statistical analysis was performed by using Stata 8.0 (www.stata.com). Total and age-grouped population estimates used as denominators for incidence calculations were obtained either from the national statistics website of the relevant country or from EU-IBIS participants (*17*). The denominator for disease incidence in infants <1 month, 1–5 months, and 6–11 months of age was estimated by dividing the number of infants (<1 year of age) by 12 and multiplying by the number of months in each age group, respectively. For non–type b encapsulated *H. influenzae* infections, we combined data on children 5–14 years of age with data on adults 15–64 years of age because serotype distribution, clinical presentation, and outcomes were similar for these 2 age groups. To estimate any increase in incidence of non–type b *H. influenzae* disease during the study period, an overdispersed Poisson regression model was fitted for the number of non–type b cases with covariates for year and country. To allow for changes in population and proportion of total *H. influenzae* isolates serotyped by country and year, these variables were included in the model as offsets. To control for differences in the collection of death data, we estimated case-fatality ratios (CFRs) by using the number of reported deaths as the numerator and all cases, including those for which outcome was not reported, as the denominator. We calculated age-adjusted odds ratios (ORs) for noncapsulated *H. influenzae* (ncHi) and Hib using logistic regression; we includled age and serotype (e.g., ncHi vs. Hib, *H. influenzae* type e [Hie] vs. *H. influenzae* type f [Hif]) as independent variables. Ages are given as medians and interquartile ranges (IQRs) and compared by using the Mann-Whitney U test. Proportions were compared by using the χ^2 test or Fisher exact test; continuous variables were compared by using Student *t* tests.

Results

During 1996–2006, a total of 14 countries reported 10,081 invasive *H. influenzae* cases, of which 2,836 (28.1%) were Hib, 690 (7.8%) were other capsulated *H. influenzae*, and 4,466 (44.3%) were ncHi. For 125 (1.2%) cases, the isolates were identified as non–type b, but complete serotyping was not performed. Capsular serotype was not available for 1,964 (19.5%) isolates, mainly because the isolate was not available for typing at the reference laboratory (e.g., where the isolate could not be recultured). The crude overall annual incidence rates for invasive Hib, ncHi, and non–type b encapsulated *H. influenzae* infections were 0.15, 0.28, and 0.036 cases per 100,000 population (Table 1). After adjusting for the proportion of isolates not serotyped in each country per year and population changes over time, we found a small but statistically significant increase in the incidence of non–type b *H. influenzae* disease (3.6% per year; 95% confidence interval [CI] 2.1%–5.2%).

After 2000, when all countries included in the study had implemented the Hib vaccine into their national im-

Table 1. Incidence of invasive Hib and non–type b *Haemophilus influenzae*, by country and year of infection, Europe 1996–2006*

Country	Incidence (no. cases)											
	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	Total
Austria	–	–	–	–	–	–	0	0.04 (3)	0.06 (5)	0.06 (5)	0.07 (6)	0.05 (19)
England, Wales	0.36 (183)	0.40 (205)	0.47 (244)	0.39 (202)	0.48 (248)	0.52 (274)	0.52 (273)	0.57 (302)	0.46 (245)	0.62 (333)	0.65 (350)	0.50 (2859)
Finland	0.29 (15)	0.29 (15)	0.57 (29)	0.35 (18)	0.64 (33)	0.83 (43)	0.23 (12)	0.54 (28)	0.40 (21)	0.73 (38)	0.52 (27)	0.49 (279)
Greece	–	–	–	–	–	–	–	0	0.01 (1)	0	0	0.00 (1)
Iceland	–	–	–	1.08 (3)	0	0	0	0	0	0	0	0.13 (3)
Ireland	0.14 (5)	0.30 (11)	0.17 (6)	0.21 (8)	0.08 (3)	0.23 (9)	0.18 (7)	0.20 (8)	0.35 (14)	0.29 (12)	0.52 (22)	0.25 (105)
Italy	0.05 (26)	0.08 (45)	0.04 (23)	0.03 (15)	0.02 (11)	0.02 (10)	0.01 (3)	0.02 (11)	0.01 (4)	0.02 (11)	0.02 (12)	0.03 (171)
Malta	–	–	–	0	0	0	0	0	0.26 (1)	0	0	0.03 (1)
The Netherlands	0.41 (64)	0.44 (68)	0.45 (70)	0.36 (56)	0.38 (61)	0.44 (71)	0.43 (67)	0.64 (103)	0.49 (80)	0.61 (99)	0.61 (99)	0.48 (838)
Norway	–	–	–	1.51 (67)	1.12 (50)	1.04 (47)	1.39 (63)	0.92 (42)	0.83 (38)	0.85 (39)	1.12 (52)	1.10 (398)
Portugal	–	–	–	0.05 (5)	0.07 (7)	0.15 (15)	0.12 (12)	0.09 (9)	0.08 (8)	0.10 (11)	0.16 (17)	0.10 (84)
Scotland	–	–	–	0	0.04 (2)	0.10 (5)	0.34 (17)	0.55 (28)	0.61 (31)	0.65 (33)	0.57 (29)	0.36 (145)
Slovenia	–	–	–	–	0.30 (6)	0.80 (16)	0.35 (7)	0.60 (12)	0.60 (12)	0.40 (8)	0.60 (12)	0.52 (73)
Total non–type b *H. influenzae* incidence	0.22 (293)	0.26 (344)	0.28 (372)	0.24 (374)	0.27 (421)	0.31 (490)	0.28 (461)	0.31 (546)	0.26 (460)	0.33 (589)	0.35 (626)	0.28 (4,976)
Hib incidence	–	–	–	–	0.12 (182)	0.14 (212)	0.23 (376)	0.21 (380)	0.15 (268)	0.13 (239)	0.09 (168)	0.15 (1,825)
Population, millions	132.5	132.8	132.9	153.9	156.3	156.9	165.6	177.4	178.5	179.8	180.6	

*Per 100,000 population. Data for Attiki, Greece (1996–2002); Germany; and Israel were not included because their surveillance was limited to pediatric cases. Hib, *H. influenza* type b.

munization programs, 7,211 *H. influenzae* cases occurred, including 2,005 (27.8%) Hib and 3,172 (44.0%) ncHi cases. Patient sex did not differ for Hib and ncHi infections (931/1,948 [47.8%]) Hib cases in female patients and 1,519/3,116 [48.7%] ncHi cases in male patients; $\chi^2 = 0.44$; $p = 0.51$). However, a higher proportion of women in the 25–44-year age group developed invasive ncHi infection: 117/165 (70.9%) for those 25–34 years of age and 93/166 (56.0%) for those 35–44 years of age, compared with 1,310/2,785 (47.0%) for the other age groups. Women 25–44 years of age who had invasive ncHi infection also were more likely than men in the same age group to have bacteremia (173/288 [60.1%] vs. 69/175 [39.4%]; $\chi^2 = 18.6$; $p<0.001$). For Hib, sex was not associated with clinical presentation for persons in any age group.

Children with Hib disease were much younger than those with ncHi (median 4.5 years [IQR 1.5–46.3 years] vs. 58.2 [IQR 6.8–76.4]) years, $p<0.0001$) (Figure). More than half of Hib cases, compared with fewer than one quarter of ncHi cases, occurred in children <5 years of age ($\chi^2 = 438$; $p<0.0001$). By contrast, only 13.7% of Hib cases occurred in persons ≥65 years of age, compared with 42.7% of ncHi ($\chi^2 = 483$; $p<0.0001$). The most common clinical diagnosis was bacteremia for both Hib and ncHi, but the median age of persons with Hib bacteremia was much lower than for that for those with ncHi bacteremia (Table 2). Hib meningitis occurred mainly in infants, whereas ncHi meningitis occurred in all age groups. Overall, ncHi was responsible for one third of all meningitis cases but accounted for 18.4% of meningitis cases in children <5 years of age and for 62.9% of persons ≥65 years of age. Hib was responsible for 84.1% of epiglottitis cases; only 6.6% were caused by ncHi. Hib pneumonia occurred mainly in adults and elderly persons; ncHi pneumonia occurred more often in children <5 years of age and in elderly persons.

In infants, the overall incidence of Hib and ncHi was similar, but incidence of the latter was much higher in the first month of life (11.4 vs. 1.2 cases per 100,000 population). In the first month, ncHi cases were more likely to occur in the first week (148/182 [81.3%]) compared with Hib (9/19 [47.4%] cases; $\chi^2 = 11.6$; $p = 0.001$); 112/148 (75.7%) of ncHi case-patients in the first week of life had bacteremia, compared with 50.0% (17/34) of those in whom ncHi occurred at 7–30 days of life ($\chi^2 = 8.8$;

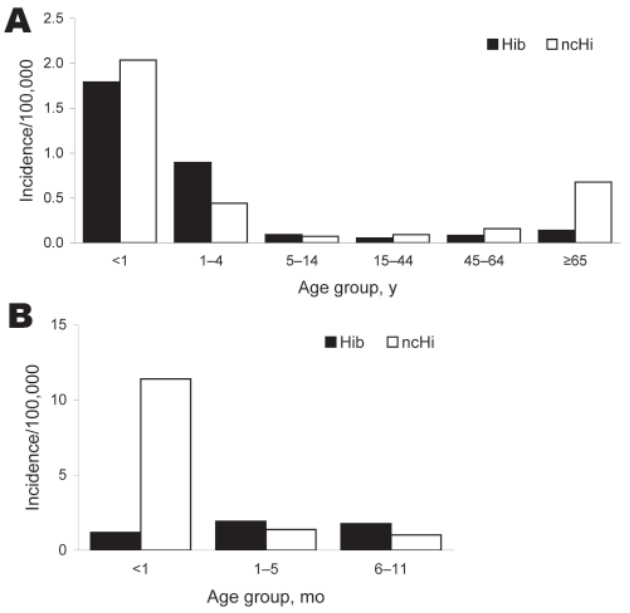

Figure. Age-specific incidence for disease caused by *Haemophilus influenzae* type b (Hib) and noncapsulated *H. influenzae* (ncHi) for all countries combined, Europe, 2000–2006. A) All age groups; B) infants <1 year of age.

p = 0.003). Infections from ncHi in infants decreased after the first month of life and remained fairly constant during the first year (Figure).

A total of 585 (8.1%) of the 7,211 persons with *H. influenzae* died; CFRs were highest for persons ≥65 years of age and for infants. We found no association between death and sex or year of infection. In most age groups, CFRs were higher for ncHi than for Hib; the largest difference was for infants (Table 3). For both Hib and ncHi, the CFR was lower for meningitis than for other clinical presentations. The age-adjusted OR for death from ncHi compared with Hib was 2.4 (95% CI 1.9–3.1, p<0.0001) overall, 3.3 (95% CI 1.5–7.5; p = 0.004) for pneumonia, and 3.3 (95% CI 1.5–7.5; p = 0.004) for bacteremia. The OR for meningitis was not significant (OR 0.85, 95% CI 0.4–1.9 years; p = 0.68).

Invasive infections caused by non–type b encapsulated *H. influenzae* were rare. Cases did not cluster by country or time, and individual serotypes or incidence, either overall or in any single country, did not increase during the 11-year study period. Of the 690 cases, Hif was the most prevalent subtype (500 [72.5%] patients) followed by Hie (143 [20.7%]) (Table 4). The overall CFR was 9.1% (63/690 patients). The CFR increased with age and was highest for bacteremia (36/292 [12.3%]) and pneumonia (15/127 [11.8%]) compared with meningitis (5/120 [4.2%]) and epiglottitis (0/10). CFR was highest for Hie infections (23/143 patients [16.1%]), particularly for persons ≥65 years of age (17/72 [23.6%]); however, none of the 22 children <16 years of age who had Hie infection died. Compared with Hif, the age-adjusted OR for death from Hie was 2.0 (95% CI 1.1–3.9; p = 0.035). All 3 Hia-related fatalities occurred in children ≤2 years who had meningitis.

The epidemiology of non–type b encapsulated *H. influenzae* varied with age. A total of 140 (20.4% of all infections) occurred in children <5 years of age. In this age group, meningitis (61 [43.6%] patients) was the most common clinical presentation and was caused by Hif (49 patients [80.3%]), Hie (7 patients [11.5%]), and Hia (5 patients [8.2%]). Children <5 years of age were more likely to have meningitis than were older children and adults (61/120 [50.8%] vs. 59/548 [10.8%]; $\chi^2 = 83$; p<0.0001). Two thirds of Hia infections (17/26 [65.4%] patients) occurred in children <5 years of age, compared with 21.2% (106/500) of Hif, 9.8% (14/143) of Hie, and 33.3% of Hic (3/9). Persons in this age group with either Hif (49/106 [46.2%] vs. 37/392 [9.4%]; $\chi^2 = 79.0$; p<0.0001) or Hie (7/14 [50.0%] vs. 15/129 [11.6%]; $\chi^2 = 14.3$; p<0.0001) infections were more likely to have meningitis, whereas older children and adults were more likely to have bacteremia and pneumonia. The CFR for children <5 years of age was lower than that for older children and adults (6/140 [4.3%] vs. 57/548 [10.4%]; $\chi^2 = 5.0$; p = 0.025).

Non–type b encapsulated *H. influenzae* infections among the 247 persons 5–64 years of age resulted mainly from Hif (17 [69.2%]) and Hie (57 [23.1%]). Most Hic (5/9 [55.6%]) and Hid (10/12 [83.3%]) infections also occurred in this age group. All serotypes were responsible for the 45 meningitis cases: Hif (26 [57.8%]), Hie (12 [26.7%]), Hid (4 [8.9%]), Hic (2 [4.4%]), and Hia (1 case [2.2%]). The CFR for this age group was 7.3% (18/247 patients), but no persons with meningitis died, compared with 12.0% (9/75 patients) and 11.5% (6/52 patients) of those with bacteremia and pneumonia, respectively.

Almost half the cases (301 [43.6%]) occurred among persons ≥65 years of age; Hif (221/301 [73.4%]) and Hie (72/301 [23.9%]) accounted for almost all cases. The overall CFR was highest for this age group (39/301 [13.0%] patients) and similar for those with bacteremia (17/102 [16.7%]), pneumonia (9/59 [15.3%]), or meningitis (2/14 [14.3%]). The CFR for Hie was 23.6% (17/72 patients), compared with 10.0% (22/221) for Hif ($\chi^2 = 8.8$; p = 0.003) for persons ≥65 years of age.

Discussion

The marked reduction in invasive Hib disease after the introduction of the Hib conjugate vaccine had prompted concerns that other *H. influenzae* serotypes, ncHi, or other respiratory pathogens might fill the ecologic niche. However, little evidence exists for a substantial or sustained increase in invasive non–type b *H. influenzae* infections (*18*). The rise in Hib incidence during 2000–2002 resulted

Table 2. Hib and ncHi cases, by diagnosis and age group, Europe 1996–2006*

Diagnosis	Age group									NR	Total	Median age, y
	<1 mo	1–5 mo	6–11 mo	<1 y	1–4 y	5–14 y	15–44 y	45–64 y	≥65 y			
Hib												
Meningitis	6 (31.6)	93 (60.4)	98 (57.6)	197 (57.4)	282 (41.2)	45 (23.0)	26 (10.0)	17 (7.1)	12 (4.4)	1 (12.5)	580 (28.9)	1.6
Epiglottitis	0	1 (0.6)	4 (2.4)	5 (1.5)	110 (16.1)	29 (14.8)	45 (17.4)	37 (15.4)	22 (8.0)	0	248 (12.4)	6.7
Cellulitis	0	7 (4.5)	18 (10.6)	25 (7.3)	15 (2.2)	3 (1.5)	0	1 (0.4)	4 (1.5)	0	48 (2.4)	1.0
OM/SA	0	4 (2.6)	5 (2.9)	9 (2.6)	24 (3.5)	4 (2.0)	4 (1.5)	6 (2.5)	1 (0.4)	0	48 (2.4)	2.1
Pneumonia	0	3 (1.9)	5 (2.9)	8 (2.3)	20 (2.9)	15 (7.7)	35 (13.5)	36 (14.9)	47 (17.2)	1 (12.5)	162 (8.1)	46.7
Bacteremia	11 (57.9)	33 (21.4)	30 (17.6)	74 (21.6)	193 (28.2)	73 (37.2)	102 (39.4)	96 (39.8)	134 (48.9)	2 (25.0)	674 (33.6)	13.6
Other	0	1 (0.6)	2 (1.2)	3 (0.9)	10 (1.5)	6 (3.1)	10 (3.9)	11 (4.6)	7 (2.6)	0	47 (2.3)	34.6
NR	2 (10.5)	12 (7.8)	8 (4.7)	22 (6.4)	30 (4.4)	21 (10.7)	37 (14.3)	37 (15.4)	47 (17.2)	4 (50.0)	198 (9.9)	39.0
All cases	19 (100.0)	154 (100.0)	170 (100.0)	343 (100.0)	684 (100.0)	196 (100.0)	259 (100.0)	241 (100.0)	274 (100.0)	8 (100.0)	2005 (100.0)	4.5
ncHi												
Meningitis	8 (4.4)	15 (13.6)	19 (19.4)	42 (10.8)	76 (22.6)	37 (24.2)	65 (14.3)	63 (13.6)	45 (3.3)	4 (20.0)	332 (10.5)	19.7
Epiglottitis	2 (1.1)	0	0	2 (0.5)	2 (0.6)	1 (0.7)	5 (1.1)	5 (1.1)	3 (0.2)	0	18 (0.6)	42.1
Cellulitis	0	0	1 (1.0)	1 (0.3)	4 (1.2)	5 (3.3)	0	0	4 (0.3)	0	14 (0.4)	11.6
OM/SA	0	0	0	0	2 (0.6)	1 (0.7)	2 (0.4)	2 (0.4)	4 (0.3)	0	11 (0.3)	56.6
Pneumonia	10 (5.5)	14 (12.7)	11 (11.2)	35 (9.0)	39 (11.6)	11 (7.2)	34 (7.5)	56 (12.1)	226 (16.7)	0	401 (12.6)	70.0
Bacteremia	129 (70.9)	53 (48.2)	46 (46.9)	228 (58.5)	151 (44.8)	74 (48.4)	236 (52.0)	218 (47.0)	759 (56.1)	10 (50.0)	1,676 (52.8)	59.6
Other	3 (1.6)	8 (7.3)	8 (8.2)	19 (4.9)	21 (6.2)	5 (3.3)	20 (4.4)	9 (1.9)	38 (2.8)	2 (10.0)	114 (3.6)	31.2
NR	30 (16.5)	20 (18.2)	13 (13.3)	63 (16.2)	42 (12.5)	19 (12.4)	92 (20.3)	111 (23.9)	275 (20.3)	4 (20.0)	606 (19.1)	61.3
All cases	182 (100.0)	110 (100.0)	98 (100.0)	390 (100.0)	337 (100.0)	153 (100.0)	454 (100.0)	464 (100.0)	1354 (100.0)	20 (100.0)	3,172 (100.0)	58.2

*Pediatric cases only for Attiki, Greece (1996–2002); Germany (1998 onward); and Israel (1996 onward). Values are no. (%) cases except as indicated. Hib, *Haemophilus influenzae* type b; ncHi, noncapsulated *H. influenzae*; NR, not recorded; OM/SA, osteomyelitis/septic arthritis.

mainly from an increase in the United Kingdom and the Netherlands; however, rates remained well below those in the prevaccine era (*19*,*20*).

Although prospective enhanced national surveillance may be incomplete (*21*), comparisons over time and across serotypes are largely valid, assuming serotyping is accurate and complete. In addition, although lower ascertainment might lead to lower estimation of the true incidence of invasive *H. influenzae* disease, it is less likely to affect the clinical presentation, age distribution, outcome, or proportion of cases due to the different serotypes. All participating countries had reference laboratories that routinely serotyped all invasive *H. influenzae* strains and participated in an external quality assurance scheme. As a result, 80.5% of 10,081 *H. influenzae* isolates identified were serotyped. The robustness of the surveillance is demonstrated by the incidence of invasive non–type b disease, which remained relatively constant over the 11-year study period despite increasing numbers of participating countries and provides further confidence that replacement disease is not occurring (*18*,*22*,*23*).

In countries with established Hib immunization programs, the incidence of ncHi is now higher than that of Hib. Unlike Hib, however, invasive ncHi infections occur mainly in neonates and elderly persons. Neonatal ncHi infections are well described but account for <5% of all neonatal invasive bacterial infections (*3*,*24*,*25*). The infection develops rapidly (usually within 48 hours after birth) and follows a fulminant course with a high CFR, particularly in preterm infants (*3*). Invasive ncHi disease in neonates also is associated with septicemia in the mother, increased complications during labor, and preterm delivery (*24*,*26*,*27*). In our study, ncHi was more common among women in

Table 3. Case-fatality rates for Hib and ncHi, by diagnosis and patient age group, Europe, 1996–2006*

Diagnosis	Age group										
	<1 mo	1–5 mo	6–11 mo	<1 y	1–4 y	5–14 y	15–44 y	45–64 y	≥65 y	NR	Total
Hib											
Meningitis	1/6 (16.7)	3/93 (3.2)	2/98 (2.0)	6/197 (3.0)	11/282 (3.9)	4/45 (8.9)	0/26 (0)	1/17 (5.9)	2/12 (16.7)	0/1 (0)	24/580 (4.1)
Epiglottitis	–	0/1 (0)	0/4 (0)	0/5 (0)	3/110 (2.7)	1/29 (3.4)	2/45 (4.4)	2/37 (5.4)	0/22 (0)	–	8/248 (3.2)
Cellulitis	–	0/7 (0)	0/18 (0)	0/25 (0)	0/15 (0)	0/3 (0)	–	0/1 (0)	0/4 (0)	–	0/48 (0)
OM/SA	–	0/4 (0)	0/5 (0)	0/9 (0)	0/24 (0)	0/4 (0)	0/4 (0)	0/6 (0)	0/1 (0)	–	0/48 (0)
Pneumonia	–	0/3 (0)	0/5 (0)	0/8 (0)	0/20 (0)	0/15 (0)	1/35 (2.9)	1/36 (2.8)	5/47 (10.6)	1/1 (100)	8/162 (4.9)
Bacteremia	0/11 (0)	2/33 (6.1)	1/30 (3.3)	3/74 (4.1)	6/193 (3.1)	2/73 (2.7)	7/102 (6.9)	4/96 (4.2)	18/134 (13.4)	1/2 (50.0)	41/674 (6.1)
Other	–	1/1 (100.0)	0/2 (0)	1/3 (33.3)	1/10 (10.0)	0/6 (0)	2/10 (20.0)	0/11 (0)	0/7 (0)	–	4/47 (8.5)
NR	0/2 (0)	0/12 (0)	0/8 (0)	0/22 (0)	0/30 (0)	0/21 (0)	1/37 (2.7)	1/37 (2.7)	1/47 (2.1)	0/4 (0)	3/198 (1.5)
All cases	1/19 (5.3)	6/154 (3.9)	3/170 (1.8)	10/343 (2.9)	21/684 (3.1)	7/196 (3.6)	13/259 (5.0)	9/241 (3.7)	26/274 (9.5)	2/8 (25.0)	88/2,005 (4.4)
ncHi											
Meningitis	0/8 (0)	1/15 (6.7)	1/19 (5.3)	2/42 (4.8)	4/76 (5.3)	3/37 (8.1)	0/65 (0)	1/63 (1.6)	2/45 (4.4)	2/4 (50.0)	14/332 (4.2)
Epiglottitis	1/2 (50.0)	–	–	1/2 (50.0)	0/2 (0)	0/1 (0)	0/5 (0)	0/5 (0)	0/3 (0)	–	1/18 (5.6)
Cellulitis	–	–	0/1 (0)	0/1 (0)	0/4 (0)	0/5 (0)	–	–	1/4 (25.0)	–	1/14 (7.1)
OM/SA	–	–	–	–	0/2 (0)	0/1 (0)	0/2 (0)	0/2 (0)	0/4 (0)	–	0/11 (0)
Pneumonia	3/10 (30.0)	0/14 (0)	1/11 (9.1)	4/35 (11.4)	4/39 (10.3)	2/11 (18.2)	4/34 (11.8)	6/56 (10.7)	40/226 (17.7)	–	60/401 (15.0)
Bacteremia	22/129 (17.1)	18/53 (34.0)	0/46 (0)	40/228 (17.5)	14/151 (9.3)	2/74 (2.7)	11/236 (4.7)	22/218 (10.1)	131/759 (17.3)	2/10 (20.0)	222/1,676 (13.2)
Other	2/3 (66.7)	6/8 (75.0)	5/8 (62.5)	13/19 (68.4)	6/21 (28.6)	1/5 (20.0)	0/20 (0)	0/9 (0)	4/38 (10.5)	1/2 (50.0)	25/114 (21.9)
NR	1/30 (3.3)	5/20 (25.0)	2/13 (15.4)	8/63 (12.7)	3/42 (7.1)	0/19 (0)	1/92 (1.1)	7/111 (6.3)	24/275 (8.7)	0/4 (0)	43/606 (7.1)
All cases	29/182 (15.9)	30/110 (27.3)	9/98 (9.2)	68/390 (17.4)	31/337 (9.2)	8/153 (5.2)	16/454 (3.5)	36/464 (7.8)	202/135 4 (14.9)	5/20 (25.0)	366/3,172 (11.5)

*Pediatric cases only for Attiki, Greece (1996–2002); Germany (1998 onward); and Israel (1996 onward). Values are no. deaths/no. cases (case-fatality rate). Hib, *Haemophilus influenzae* type b; ncHi, noncapsulated *H. influenzae*; OM/SA, osteomyelitis/septic arthritis; NR, not recorded.

the 25–44-year age group, suggesting that childbearing-aged women may be at increased risk for invasive ncHi infections. This finding may reflect increased exposure, for example, because of contact with children or increased susceptibility, such as in pregnancy. In older children and adults who develop invasive ncHi infections, studies have reported that more than half the case-patients had serious predisposing medical conditions, such as chronic respiratory disease and impaired immunity (*3*–*7*). Unfortunately, because clinical information collected for individual cases in our study was limited, we could not further elucidate possible risk factors for invasive infections caused by the different serotypes.

Infection from non–type b encapsulated *H. influenzae* is extremely rare and mostly caused by Hif and Hie. Other population-based studies also have reported a predominance of Hif and, to a lesser extent, Hie among non–type b encapsulated *H. influenzae* in adults and children (*3*,*6*,*28*). The clinical presentations of both Hif and Hie disease are almost identical and similar to that of ncHi infections in that almost half the cases occurred among persons ≥65 years of age who usually had bacteremia and pneumonia (*6*,*7*). Although 44% of Hif infections occurred among persons ≥65 years of age, compared with 21% among children <5 years, the incidence was almost the same in the 2 age groups. Hif and Hie have considerably restricted genetic diversity, and most infections are caused by a few strains that may be intrinsically more pathogenic than noninvasive strains (*29*). Other studies have reported that, as with ncHi, 60%–80% of persons with invasive Hif disease and Hie had underlying conditions that predisposed them to opportunistic infections (*3*,*6*,*9*–*11*,*25*,*30*–*32*).

Table 4. Epidemiology, diagnosis, and outcome of invasive non–type b *Haemophilus influenzae* infections, by serotype and age group, Europe, 1996–2006*

Data	Hia, n = 26	Hic, n = 9	Hid, n = 12	Hie, n = 143	Hif, n = 500	Total, N = 690
Diagnosis, no. (%) cases						
Bacteremia	5 (19.2)	1 (11.1)	0	44 (30.8)	157 (31.4)	207 (30.0)
Pneumonia	3 (11.5)	3 (33.3)	2 (16.7)	28 (19.6)	91 (18.2)	127 (18.4)
Meningitis	6 (23.1)	2 (22.2)	4 (33.3)	22 (15.4)	86 (17.2)	120 (17.4)
Other	6 (23.1)	0 (0)	1 (8.3)	30 (21.0)	87 (17.4)	124 (18.0)
Unknown	6 (23.1)	3 (33.3)	5 (41.7)	19 (13.3)	79 (15.8)	112 (16.2)
Total	26 (100.0)	9 (100.0)	12 (100.0)	143 (100.0)	500 (100.0)	690 (100.0)
Median age at disease onset, y (IQR)	2.1 (0.8–43.7)	52.0 (1.9–53.3)	36.5 (14.4–47.0)	65.3 (34.0–8.3)	61.0 (12.7–75.2)	60.7 (15.4–75.1)
Incidence/million cases (total no. cases) by age group, y						
<5	0.12 (17)	0.02 (3)	0	0.10 (14)	0.78 (106)	1.02 (140)
5–64	0.00 (4)	0.00 (5)	0.01 (10)	0.04 (57)	0.12 (171)	0.17 (247)
≥65	0.02 (5)	0.00 (1)	0.01 (2)	0.25 (72)	0.77 (221)	1.04 (301)
All age groups	0.01 (26)	0.00 (9)	0.01 (12)	0.08 (143)	0.26 (500)†	0.36 (690)†
No. cases (%) by age group, y						
<5	17 (65.4)	3 (33.3)	0	14 (9.8)	106 (21.2)	140 (20.3)
5–64	4 (15.4)	5 (55.6)	10 (83.3)	57 (39.9)	171 (34.2)	247 (35.8)
≥65	5 (19.2)	1 (11.1)	2 (16.7)	72 (50.3)	221 (44.2)	301 (43.6)
All age groups	26 (100.0)	9 (100.0)	12 (100.0)	143 (100.0)	500 (100.0)†	690 (100.0)†
No. (%) deaths by age group, y						
<5	3 (17.6)	0	–	0	3 (2.8)	6 (4.3)
5–64	0	1 (20.0)	0	6 (10.5)	11 (6.4)	18 (7.3)
≥65	0	0	0	17 (23.6)	22 (10.0)	39 (13.0)
All age groups	3 (11.5)	1 (11.1)	0	23 (16.1)	36 (7.2)†	63 (9.1)†
Median age at death, y (IQR)	0.50 (0.47–2.3)	55.0‡	–	70.0 (63.8–88.9)	72.3 (55.9–83.4)	70.5 (51.7–83.7)

*Hia, *H. influenzae* type a; Hic, *H. influenzae* type c; Hid, *H. influenzae* type d; Hie, *H. influenzae* type e; Hif, *H. influenzae* type f; IQR, interquartile range.
†Ages were not known for 2 persons with Hif infection.
‡Only 1 death was caused by this serotype.

In contrast to Hif and Hie, invasive Hia infections were similar to Hib infection in that they occurred mainly in young children who often had meningitis (*2*). In our study, the incidence of Hia in children <5 years of age (0.12/million) was much lower than that reported in Navajo and White Mountain Apache children <5 years of age (20 cases/100,000 population) (*33*), Alaska Native children <2 years of age (21/100,000) (*34*), and northern Canadian aboriginal children <2 years of age (102/100,000) (*34*). The same populations are also highly susceptible to invasive Hib disease (*35*,*36*). Hia and Hib have the most closely related capsules (*37*) and a similar degree of genetic diversity (*29*).

Infections caused by Hic and Hid are rare and have low CFRs, suggesting that they may not be particularly virulent. There is a paucity of information on infections caused by these serotypes, even in the form of individual case reports. Our data suggest that these invasive Hic and Hid infections are more common in adults. A recent US study reported that, of 770 cases of invasive *H. influenzae* disease during 1996–2004 in Illinois, 3 (43%) of 7 Hic, and 4 (67%) of 6 Hid cases occurred in persons 18–64 years of age (*28*).

That CFR from invasive Hib disease remains low and similar to that reported in other industrialized countries is reassuring, even though it has not changed substantially from the prevaccine era (*2*,*38*). In contrast, CFRs for ncHi and non–type b encapsulated *H. influenzae* were significantly higher than for Hib. The CFRs in our study should be considered a minimum because we cannot be sure that all deaths would be reported to the surveillance systems, particularly if death occurred a considerable time after infection. Other studies with more active follow-up in adults with ncHi infections have reported CFRs of 13%–20% (*28*) and up to 29% within 1 month after infection (*7*). Although the high CFRs associated with early-onset neonatal ncHi is well described (*3*), our finding of such high CFRs in infants was unexpected. Whether these infants had any underlying medical conditions that predisposed them to death or the organisms causing infection in this age group are more virulent is not known. The CFR for invasive non–type b encapsulated *H. influenzae* infections was also higher than for Hib and comparable to the 15%–30% reported in other studies (*7*,*28*). The higher CFR for invasive ncHi infections among elderly persons and persons with other clinical diagnoses (Table 3) most likely results from a higher prevalence of underlying medical conditions predisposing them to opportunistic infections. In the latter group, for example, ncHi was often isolated from uncommon sterile sites, such

as peritoneal and pericardial fluid, renal and spleen biopsy specimens, and brain abscesses, suggesting that such persons may have serious underlying medical conditions at the time of infection. Underlying medical conditions also may explain why CFRs may be higher for persons with invasive Hie infections; the small number of Hie cases compared with Hib, ncHi, or Hif suggests that this serotype is not particularly virulent. Other studies have reported higher CFRs for Hie than for Hif, particularly for elderly persons (*28*).

Thus, despite the reduction in Hib disease, continued surveillance is needed for all *H. influenzae* infections across all age groups to assess the long-term effectiveness of Hib vaccination, rapidly detect unexpected population effects and potential changes in circulating strains, and monitor changes in the epidemiology of invasive *H. influenzae* disease. Further studies are needed to define more clearly host and pathogen risk factors for invasive *H. influenzae* infection and factors associated with death.

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Acknowledgments

We thank Nick Andrews for his help with statistical analysis.

This study was funded by European Union Biomedicine and Health Research Programme II Reference BMH4960984 (1996–1999), EU DG SANCO agreement no. VS/1999/3504 99CVF4-031 (1999–2001), EU DG SANCO (European Union Directorate-General for Health and Consumer Protection) agreement no.2003202 (2001–2003), EU DG SANCO agreement no.2003202 (2003–2006). Dr Ladhani's salary was funded through a competitive 2-year fellowship awarded by the European Society for Paediatric Infectious Diseases.

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Vaccine Preventability of Meningococcal Clone, Greater Aachen Region, Germany

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Emergence of serogroup B meningococci of clonal complex sequence type (ST) 41/44 can cause high levels of disease, as exemplified by a recent epidemic in New Zealand. Multiplication of annual incidence rates (3.1 cases/100,000 population) of meningococcal disease in a defined German region, the city of Aachen and 3 neighboring countries (Greater Aachen) prompted us to investigate and determine the source and nature of this outbreak. Using molecular typing and geographic mapping, we analyzed 1,143 strains belonging to ST41/44 complex, isolated from persons with invasive meningococcal disease over 6 years (2001–2006) from 2 German federal states (total population 26 million) and the Netherlands. A spatially slowly moving clone with multiple-locus variable-number tandem repeat analysis type 19, ST42, and antigenic profile B:P1.7–2,4:F1–5 was responsible for the outbreak. Bactericidal activity in serum samples from the New Zealand MeNZB vaccination campaign confirmed vaccine preventability. Because this globally distributed epidemic strain spreads slowly, vaccination efforts could possibly eliminate meningococcal disease in this area.

Our work describes the epidemiology of invasive meningococcal disease (IMD) caused by meningococci of clonal complex (cc) 41/44 in the Netherlands and the 2 bordering German states Lower Saxony and North-Rhine-Westphalia during 2001–2006. *Neisseria meningitis* is a gram-negative bacterium that occasionally causes invasive disease in humans, primarily meningitis or sepsis (*1*). Notwithstanding low incidence rates in most industrialized countries, IMD remains a serious public health problem because of its predilection for affecting young persons and its ≈10% death rate despite antimicrobial drug treatment. In contrast to the meningitis belt in Africa, where epidemic waves cause incidence rates up to 300 cases/100,000 population (*2*), epidemics or case clusters are rare in industrialized countries (*3*). Meningococci are antigenically diverse bacteria that can be divided into 12 serogroups by variation of their polysaccharide capsules. Despite increased findings of serogroup C meningococci in several countries, serogroup B has clearly controlled the epidemiology of IMD in western Europe for the past 20 years. Dominance of serogroup B has further been compounded by numerous vaccination campaigns with polysaccharide C conjugate vaccine leading to a decline in serogroup C disease (*4*). Unfortunately, the serogroup B polysaccharide is an unsuitable vaccine antigen because of poor immunogenicity. Despite substantial progress in the development of vaccines based on membrane-associated antigens (*5*,*6*), a universal vaccine against meningococci has yet to be licensed.

Typing of *N. meningitidis* is critical for tracking transmissions and recognition of disease clusters. In recent years, focus has shifted to portable molecular typing methods with high discriminatory power. The preferred method for sequence-based typing of meningococci is multilocus sequence typing (MLST) (*7*), which enables identification of strains belonging to hypervirulent clonal complexes responsible for most cases of the invasive disease (*8*). MLST is complemented by antigen sequence typing of the variable regions of the outer membrane proteins PorA and FetA (*9*). Moreover, multiple-locus variable-number tandem repeat

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DOI: 10.3201/eid1603.091102

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analysis (MLVA), which shows slightly higher discriminatory ability than MLST (*10*), represents a recent addition to the arsenal of portable typing methods for *N. meningitidis*.

Differences in the antigenic makeup of meningococcal clonal complexes (cc) (*11*) likely influence reported disparities in spatiotemporal spread. Whereas strains belonging to the multilocus sequence type (ST) 5 complex (cc5/subgroup III) (*12*) and ST11 complex (cc11/ET-37 complex) (*13*) depend on migration to survive, strains of the ST41/44 complex (cc41/44/lineage 3) have been described as causing stationary and persistent hyperendemic disease, as exemplified by the New Zealand serogroup B epidemic, which lasted more than a decade (*14*).

cc41/44 is a large hypervirulent complex that revolves around 2 STs instead of 1 central ST, namely ST41 and ST44 (*15*). It was first described in the Netherlands in the 1980s (*16*), where it caused a substantial increase in disease incidence (*17*,*18*). Subsequently, this lineage was reported in Belgium in the early 1990s (*19*), then New Zealand since 1991 (*14*). In New Zealand an epidemic with incidences up to 17.4 cases/100,000 population in 2001 prompted an immunization campaign with custom made outer-membrane-vesicle vaccine MeNZB (Novartis Vaccines and Diagnostics, Siena, Italy) (*20*).

By using cluster detection software SaTScan (www.satscan.org) (*21*) for laboratory surveillance of IMD at the German Reference Center for Meningococci (*22*), we showed spatial concentrations of meningococci with antigen sequence type B:P1.7–2.4:F1–5 (serogroup B, PorA VR1 7–2, PorA VR2 4, and FetA VR 1–5), strongly associated with cc41/44 (*11*), around the German city of Aachen near its border with the Netherlands. The annual incidence rate rose to 3.1/100,000 in 2005 among a population of 1.1 million living in Aachen and 3 neighboring counties (Greater Aachen; Figure 1).

We mapped the epidemiology of IMD caused by cc41/44 meningococci in the Netherlands and 2 neighboring German states, Lower Saxony and North-Rhine-Westphalia, during 2001–2006. Furthermore, we characterized the clone responsible for the upsurge of IMD in Greater Aachen.

Materials and Methods

Bacterial Strains

All *N. meningitidis* strains analyzed in this study were isolated from patients with IMD. One isolate was included for each patient. During 2001–2006, a total of 239 strains collected by NRZM and 904 isolates collected by the Neth-

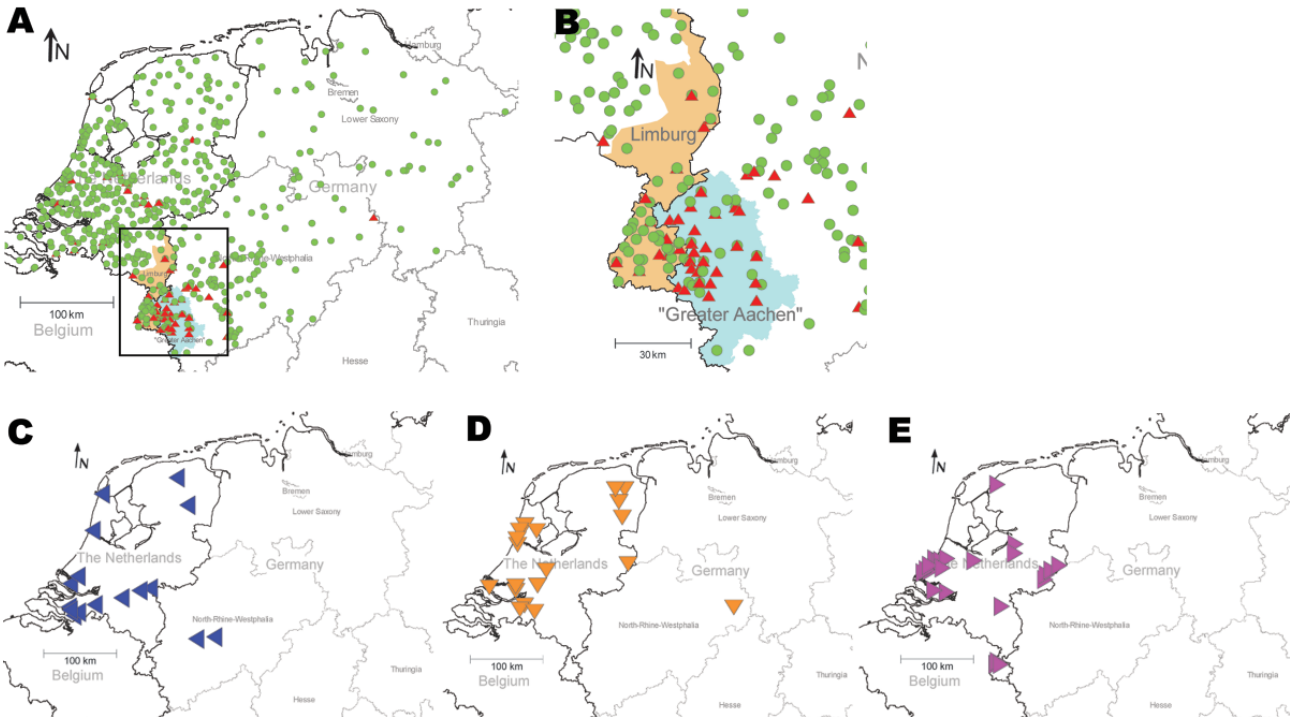

Figure 1. Distribution of cc41/44 *Neisseria meningitidis* strains in Germany and the Netherlands during 2001–2006 with positively associated multiple-locus variable-number tandem repeat analysis (MLVA) and multilocus sequence types (MLST). A) Distribution of MLVA type (MT) 19/MLST (ST) 42 strains (red triangles). Full green circles represent non–MT19/ST42 strains. Black rectangle delineates the area magnified in panel B. B) Area encompassing Limburg (orange shading) and Greater Aachen (blue shading). C–E) Spatial distribution of other overrepresented MT/ST variants: MT27/ST40 (blue triangles); MT30/ST40 (orange triangles); MT78/ST1374 (purple triangles).

erlands Reference Laboratory for Bacterial Meningitis, Academic Medical Center, Amsterdam, North Holland, the Netherlands) were included in the study. Only strains of serogroup B with positive amplification of cc41/44-specific restriction-modification system *Neisseria meningitis* (*Nme)* SI (*17*) and subsequent confirmation of cc41/44 by MLST were included. In addition, 51 serogroup B, *Nme*SI–positive strains obtained from the Netherlands in 1985 were analyzed as a historic reference. NZ98/254 is the meningococcal strain used to make MeNZB (Novartis Vaccines and Diagnostics) (*20*).

Typing of Meningococci

MLST (*7*) and antigen sequence typing of PorA (*23*) and FetA (*24*) were performed according to published protocols. MLVA targeting 8 loci was performed according to the method described by Schouls et al. (*10*). However, nomenclature of repeat profiles has been changed since their original description; current MLVA types (MTs) and conversion tables are available from www.mlva.net. Unique combinations of serogroup, PorA variable region 1 (VR1), PorA variable region 2 (VR2), and variable region of FetA (FetA VR) were termed fine types. Simpson diversity indices (DIs) of the above typing schemes used alone or in combination were calculated as outlined by Hunter and Gaston (*25*). Ninety-five percent confidence intervals (CIs) of DI were determined by using the percentile bootstrap method after 1,000 replicates implemented in the package boot, written by A. Canty and B. Ripley for R (R Foundation for Statistical Computing, Vienna, Austria), version 2.8.0 (www.r-project.org).

Spatiotemporal Data

Geographic coordinates (map datum World Geodetic System [WGS] 84) were derived from German and Dutch postal codes. Yearwise categorization of data was based on the dates of sampling or dates of entry if sampling information was not available. Maps (Figure 1) were generated by using Regiograph 8 (GfK GeoMarketing GmbH, Bruchsal, Germany). Yearwise spatial densities of MT19/ST42 strains in the study area (Figure 2) were calculated by using Spatstat, version 1.15–1, created by A. Baddeley and R. Turner for the statistical environment R.

Statistical Analyses

Covariation among MTs and STs was assessed by using the Jaccard similarity coefficient (J), as described by Rhee et al. (*26*), with slight modifications. Briefly, for any combination of MT and ST, the J coefficient is the ratio between the number of strains belonging to the combination in question divided by the number of strains sharing either MT or ST. It is calculated as $J = N_{VS}/(N_{VS} + N_{V0} + N_{0S})$, where N_{VS} represents the number of strains with a certain MT and ST, N_{V0} is the number of strains with the same MT but another ST, and N_{0S} is the number of strains with the same ST but another MT. Observed J and expected Jaccard coefficients (J_{EXP}) were compared for each combination, assuming random coupling of types. J_{EXP} was calculated as the mean J coefficient after 2,000 random rearrangements of the MT and ST vector (consisting of Boolean values indicating presence or absence of types). p values testing the equality of J and J_{EXP} for any combination of MT and ST were derived by using an inverse quantile function based on the distribution of 10^5 bootstrap replicates. We used the Holm method to control the familywise error rate for multiple hypothesis testing (*27*). The Pearson χ^2 test was calculated with R.

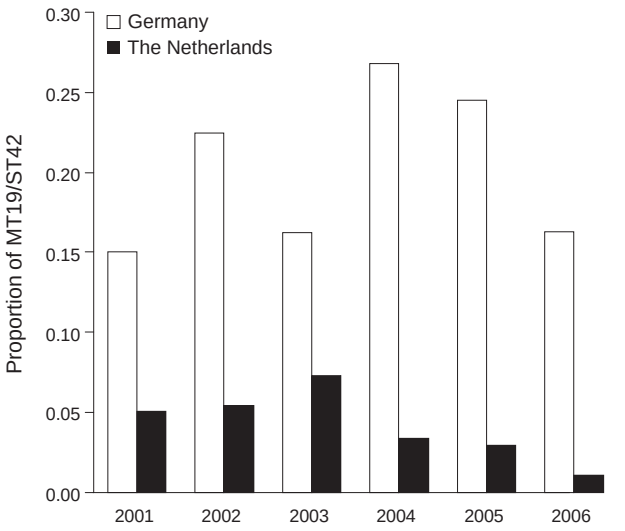

Figure 2. Temporal progression of the proportion of MT19/ST42 meningococcal strains in the Netherlands and the German study region (North-Rhine-Westphalia and Lower Saxony), 2001–2006.

Serum Bactericidal Assay

German strain DE9686 (B:P1.7–2,4:F1–5:ST42: MT19) isolated in 2004 from a patient of IMD in Aachen was used as the target strain in a validated serum bactericidal assay (*28*). Because the serum complement source used in the New Zealand trials contained interfering antibodies against strain DE9686, an alternative serum complement source for strain DE9686 was found from a range of adult volunteers. Prevaccination and postvaccination serum samples from 20 persons, 18 months–12 years of age, who had been vaccinated with 3 doses of MeNZB vaccine (Novartis Vaccines and Diagnostics) during the New Zealand trials, were tested against the German target strain, DE9686 by using the new serum complement source. Interpolated titer values were measured by using a formula that calculates the level of antibodies on the basis of percentage kill immediately on either side of the 50% cutoff (*28*).

Results

Performance of Typing Methods

All 1,143 strains were tested by MLVA, MLST, and antigen sequence typing. Fine typing, i.e., serogroup, PorA VR1, PorA VR2, and FetA VR type showed 195 unique types, which translates to a low DI of 0.752 (95% CI 0.726–0.778). MLVA and MLST distinguished 232 (DI 0.942, 95% CI 0.933–0.948) and 222 (DI 0.893, 95% CI 0.879–0.905) types, respectively, confirming the higher discriminatory ability of MLVA when compared with MLST. Both neutral typing methods (MLST and MLVA) provided higher discrimination than antigen sequence typing. Finally, combination of MLVA and MLST yielded 504 unique MLVA-MLST (MT-ST) types, demonstrating the extremely fine-grained resolution (Simpson's index 0.985, 95% CI 0.981–0.987) attained for the binational collection of cc41/44 *N. meningitidis* strains.

Covariation and Spatial Pattern of MLVA-MLST Combinations

Covariation was computed for MTs and STs that were identified at least 10 times. The average J coefficient for observed combinations was low (0.06), suggesting a limited overlap between MLVA and MLST. After controlling the familywise error rate at <0.01, four MT-ST combinations showed marked positive association, indicating recent clonal expansion: MT19/ST42, MT27/ST40, MT30/ST40, and MT78/ST1374. In contrast, 3 combinations occurred significantly less frequently than expected: MT27/ST41, MT30/ST41, and MT78/ST41 (Table 1). Geographic coordinates were available for 1,102 (96.4%) of 1,143 strains. Two of the positively linked MT-ST combinations showed evidence for clustering: MT78/ST1374 around the Dutch city of Den Haag and, more explicitly, MT19/ST42 in Greater Aachen (Figure 1, panels A and B).

Clustering of MT19/ST42 Meningococci

Strains with MT19/ST42 occurred almost exclusively on the German side of Greater Aachen: of 50 German MT19/ST42 strains, only 8 were isolated from outside this region, all but 1 occurred within 100 km of Aachen (Figure 1, panel B, $p<2.2 \times 10^{-16}$, χ^2 test). A similar, albeit less marked concentration, was observed for the Netherlands regarding the province of Limburg: 15 of 37 MT19/ST42 (40.1%) strains with corresponding regional data originated from this province, compared with 97 (11.7%) of 826 other cc41/44 isolates ($p = 1.2 \times 10^{-6}$, χ^2 test). From a total of 91 MT19/ST42 strains, 81 (89.0%) were B:P1.7–2.4:F1–5. In contrast to the 41 Dutch MT19/ST42 isolates, which displayed 7 different fine types with 76% dominance of B:P1.7–2.4:F1–5, all 50 German MT19/ST42 isolates were B:P1.7–2,4:F1–5. Mean annual incidence rates of MT19/ST42 per 100,000 population in the Netherlands, the German states Lower Saxony and North-Rhine Westpahlia (including Greater Aachen), and Greater Aachen were 0.04, 0.03, and 0.63, respectively. In conclusion, most of type MT19/ST42 strains were isolated from Germany (50/91 strains), where they displayed a higher degree of clustering and antigenic uniformity.

Temporal Trends and Migration of the Outbreak Strain

The total number of cc41/44 isolates declined from 257 in 2001 to 137 in 2006. The proportion of MT19/ST42 strains was higher in the German region in every year from 2001 to 2006 and peaked at 0.27 in 2004 (Figure 2. This clone was the most common MT-ST combination in the German region during 2001–2006, as opposed to the Netherlands, where the most numerous combinations per year were MT19/ST41 (2001), MT19/ST42 (2002), MT19/ST41 (2003), MT18/ST41 (2004), MT18/ST41 (2005), and MT19/ST41 together with MT18/ST41 (2006). Furthermore, the proportion of MT19/ST42 in the German area never fell below 0.15, whereas in the Netherlands it decreased to 0.01 in 2006 (Figure 3) because of slow eastward migration from the Dutch province of Limburg toward Greater Aachen (Figure 3). A historic sample from the Netherlands from 1985 yielded 1 MT19/ST42 strain out of 51 (proportion 1.96%, 95% CI 0.00–11.79), indicating either reoccurrence of this type over >2 decades or independent reassociation of alleles.

Table 1. Positively and negatively correlating MLVA-MLST pairs, *Neisseria meningitis* clone, Greater Aachen Region, Germany, 2001–2006*

Association	MT	ST	N	N_{EXP}	N (MT)	N (ST)	J	J_{EXP}	p value†
Positive	19	42	91	38	209	209	0.2783	0.1013	0
	30	40	19	3	38	82	0.1881	0.0235	0
	78	1374	25	2	54	39	0.3676	0.0199	0
	27	40	18	4	59	82	0.1452	0.032	0.00002
Negative	27	41	3	16	60	292	0.0086	0.0462	0
	30	42	1	7	38	209	0.0041	0.0287	0
	78	41	4	14	54	292	0.0117	0.0416	0.00004

*MLVA, multilocus variable tandem repeat analysis; MLST, multilocus sequence type; MT, MLVA type; ST, multilocus-sequence type, N, no. strains with MT and ST; N_{EXP}, expected number of strains with MT and ST; N (MT), no.strains with MT; N (ST), no. strains with ST; J, Jaccard index; J_{EXP}, expected Jaccard index
†2-tailed.

Age Distribution of Case-Patients

Age information was available for 1,140 of 1,143 (99.7%) case-patients. IMD caused by MT19/ST42 occurred more commonly among patients >10 years of age ($p = 4.2\times 10^{-4}$, χ^2 test). A plot of the age distribution of cases due to meningococci of cc41/44 illustrates a bimodal pattern considered typical for IMD (Figure 4). Nevertheless, cases caused by clone MT19/ST42 disproportionately affected adolescents, with no difference between the Netherlands and Germany ($p = 0.90$, χ^2). This positive shift is consistent with an epidemic age pattern described in the 1970s during an epidemic wave of meningococcal disease in Finland (*29*) and during the 1980s in the Netherlands (*30*).

Serum Bactericidal Antibody Responses against DE9686

Typing methods (MLVA, MLST, "fine typing") could not distinguish NZ98/254 from DE9686 (B:P1.7–2.4:F1–5:ST42:MT19). Serum bactericidal antibody responses of persons vaccinated with MeNZB suggested protective levels (i.e., ≥8) in all serum samples. These samples were tested in a serum bactericidal assay with DE9686 as a target strain. The test determines the maximal dilution at which killing activity of tested serum can be observed (Table 2).

A 4-fold rise in titer was observed in the postvaccination sample for all (10/10) toddlers (18–24 months of age) and 8 of 10 schoolchildren (8–12 years of age). Only titers in 2 persons with the highest prevaccination titers (189 and 229) rose <4-fold after vaccination with MeNZB (Novartis Vaccines and Diagnostics).

Discussion

Our main goals were to identify the clone causing the rise in incidence rate in Greater Aachen and to elucidate whether increased disease activity in Germany represented local emergence or cross-border spread from the Netherlands, which had experienced a steep rise in IMD caused by cc41/44 since 1980 (*18*). Tracking of variants within cc41/44 necessitated a high level of discrimination, achieved by the combination of typing methods MLVA and MLST (DI 0.985). Geographic mapping showed it was only the latter pairing of techniques that sharply delineated a spatial accumulation of MT19/ST42 meningococci in the region that had seen increase of disease rate (Figure 1). The spread of the outbreak clone differed upon introduc-

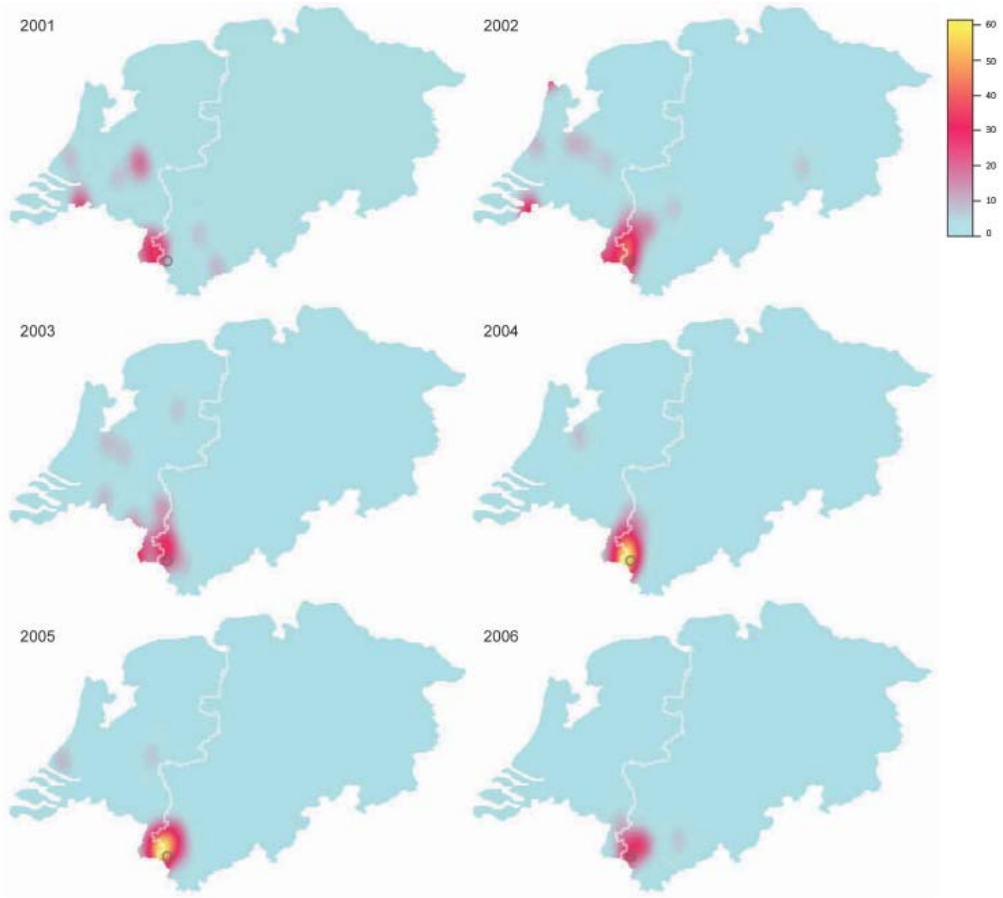

Figure 3. Yearwise spatial distribution of MT19/ST42 strains within the study region in Germany during 2001–2006. Color-coded values represent estimates of the intensity function underlying the point pattern data. Gray circle marks the city of Aachen; the white line represents the border between the Netherlands and Germany.

tion into Germany, where it caused high levels of disease in a confined area. The distinct level and high spread of disease may suggest the presence of regionally specific, as yet unknown, factors contributing to its emergence. Because behavior-related risk factors promote acquisition of IMD in adolescents (*31*), locally differing traditions in Germany, possibly related to carnival festivities in seasons with high incidence (*32*), may have contributed to the outbreak. Nevertheless, clustering was also present, albeit less abundantly, in the Dutch province of Limburg. Higher antigenic diversity and discrete eastward motion indicate the clone's longer history in the Netherlands, where it had failed to cause an epidemic, possibly due to population immunity elicited by long-lasting exposure to related variants of cc41/44 since the 1980s.

Although clusters of cases tend to be short-lived in industrialized countries (*33*), the geographic concentration of the outbreak clone was observed during the whole study period (Figure 3). This spatial stability might be promoted by the clone's more efficient evasion of induction of mucosal immunity. Notably, antigenic variation of the outbreak clone was limited and dominated by B:P1.7–2.4:F1–5 (89%). Lower antibody avidity observed after vaccination with OMVs containing P1.7–2.4 (*34*) suggests that this PorA-type evokes a less potent immune response, possibly leading to decreased protection against acquisition of carriage. Studies confirming this hypothesis, however, have not been published. Moreover, the higher diversity unveiled by neutral typing techniques compared with antigen sequence typing could suggest positive selection for strains achieving immune escape because of their antigenic profile.

There was a significant shift toward older age of patients infected by MT19/ST42 meningococci, consistent with observations before and during epidemics (*29*,*30*). A recent report demonstrates an overrepresentation of meningococci harboring the meningococcal disease associated island among young adults with IMD, possibly indicating its contribution to invasive disease in this age group (*35*). Frequent isolation of presumably more virulent meningococci, such as MT19/ST42, from adolescents might be explained by the hypotheses that 1) fewer virulence determinants are required to cause invasion in infants, hence strains of lower invasiveness are recovered in higher proportions among them, and 2) invasiveness represents a smaller penalty for highly transmissible strains in persons with abundant social contacts (*36*), leading to their preferential circulation among older age groups.

In an analysis of meningococci across several clonal complexes, Schouls et al. obtained similar groupings by MLVA and MLST (*10*). To identify type pairs deviating from their expected occurrence within strains of this study, which pertained to a single clonal complex (cc41/44), we computed the degrees of overlap (represented by J coefficients) of observed combinations between MTs and STs. The mean overlap was low (0.06), and the number of most combinations did not differ significantly from the expected, suggesting random association in most types and highlighting the complementary nature of MLST and MLVA for cc41/44. The added value of combining these typing methods is also reflected in the significantly higher DI attained. Positively associated combinations could reflect linkage disequilibrium in concordance with an epidemic population concept (*37*), which attributes disequilibrium to transient multiplication of successful variants doomed to dissipate within years, secondary to recombination. Nevertheless, recovery of MT19/ST42 over >20 years favors concepts that accommodate the observed excessive stability, e.g., proposed in models including interstrain competition (*36*). On the other hand, strains with a negative association might indicate low epidemic potential. All sequence types recovered from covariation analysis (ST40, ST42, and ST41, ST1374) represent (sub)group founders within cc41/44, attributed higher transmissibility and fitness due to persistent recovery in both carrier and invasive collections (*36*). Although observed presence of founder STs could be due to biased selection of STs for covariation analysis (only types occurring at least 10 times were included), the clear underrepresentation of some MTs belonging to mentioned STs suggests that at least virulence within these founder STs is not equally distributed (Table 1).

The following observations support the hypothesis that meningococci of the clone MT19/ST42 command exceptional epidemic potential: dramatic spatial concentration displayed in Greater Aachen, concurrent rise of

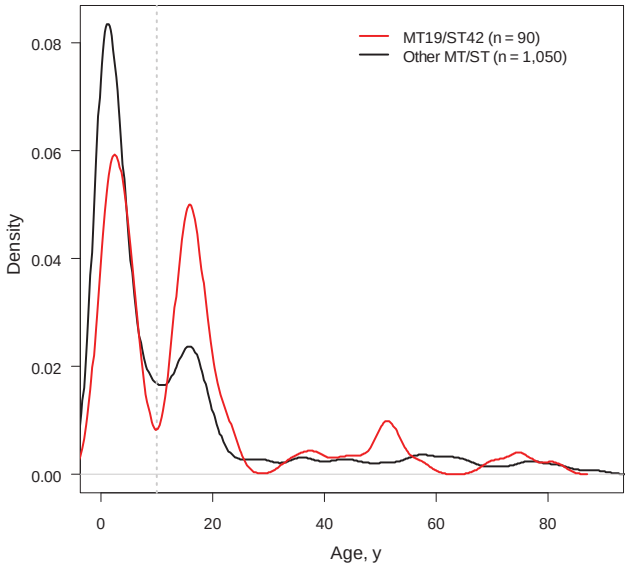

Figure 4. Kernel density plots of age distribution of MT19/ST42 case-patients compared with the rest of the ST41/44 complex. The vertical gray line indicates 10 years.

Table 2. Serum bactericidal antibody titers before and after vaccination with MeNZB against German strain DE9686, Greater Aachen Region, Germany, 2001–2006*

Age group	No. vaccinees	Before	After	4-fold rise	≥8 post
8–12 y	1	12	1,727	←	←
	2	3	104	←	←
	3	189	406	–	←
	4	50	296	←	←
	5	229	729	–	←
	6	23	175	←	←
	7	9	84	←	←
	8	2	213	←	←
	9	2	77	←	←
	10	2	55	←	←
18–24 mo	11	2	214	←	←
	12	4	45	←	←
	13	2	109	←	←
	14	7	92	←	←
	15	2	306	←	←
	16	6	431	←	←
	17	2	216	←	←
	18	3	44	←	←
	19	3	218	←	←
	20	2	171	←	←

*– represents cases where conditions 4-fold rise and ≥8 post are false. Arrows represent cases where conditions 4-fold rise and >8 post are true.

incidence in the area of clustering, and age-shift to older patients. Tendency to affect older persons has also been noted during epidemics (*29*,*30*) and in strains carrying a temperate bacteriophage associated with higher pathogenic potential (*35*). In addition, vaccine strain NZ98/254, which was used for generation of New Zealand's MeNZB (Novartis Vaccines and Diagnostics) (*38*), was not distinguishable from German MT19/ST42 strains, demonstrating the clone´s emergence on separate continents.

By using paired serum samples from vaccinated toddlers and schoolchildren (Table 2), we were able to show a striking similarity between the serum bactericidal antibody responses induced by the German epidemic strain and results obtained in New Zealand against NZ98/254 after vaccination of toddlers and 8–12 years of age (*39*,*40*). Similar to the New Zealand situation, a protective vaccine effect was strongly supported by the serum bactericidal antibody responses induced by the German strain. Moreover, because the VR2 epitope of PorA is the major target for immune response elicited by MenZB (*38*), protection against most of the analyzed cc41/44 strains (58% have VR2 type 4) can be assumed.

Although the marked concentration of meningococci with fine type B:P1.7–2.4:F1–5 continues to exist in western North-Rhine-Westphalia (www.episcangis.org), incidence rates have been decreasing since 2005; this decrease has suspended plans to implement a vaccine campaign. Nevertheless, the New Zealand experience (*14*) suggests that this clone may attain high-level endemicity, and continued close surveillance remains a task of high priority. Should regional incidences rise again, implementation of an immunization campaign with MeNZB should be considered.

Although we studied a large area involving 2 bordering countries, our study is limited. We did not include isolates from asymptomatic carriers because of the logistic difficulties related to the collection of representative carriage samples that preceded or temporally coincided with outbreaks. Furthermore, the sampling period only covered 6 years with 1 historic reference year because of the large number of strains. Finally, representative population immunity data were not available. Our study does, however, pave the ground for future epidemiologic and experimental work aimed at confirming the distinct pathogenic potential of MT19/ST42 meningococci and unraveling the circumstances leading to their spatially distinct occurrence.

We tracked an outbreak clone that was causing considerable disease activity on the border of 2 industrialized European countries by using highly discriminatory and portable typing techniques. These techniques could guide and improve the targeting of public health efforts, which may include vaccination, if incidence rates in North-Rhine-Westphalia begin to rise again.

M.F. and A.v.d.E. participate in the Sixth Framework Programme of the European Commission, Proposal/Contract no. 512061 (Network of Excellence European Virtual Institute for Functional Genomics of Bacterial Pathogens; www.noe-epg.uni-wuerzburg.de).

The project was funded in part by a grant of the Robert Koch-Institute ref. 3/6400/2008 to J.E., M.F., and U.V

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Use of Avian Bornavirus Isolates to Induce Proventricular Dilatation Disease in Conures

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Avian bornavirus (ABV) is a newly discovered member of the family *Bornaviridae* that has been associated with the development of a lethal neurologic syndrome in birds, termed proventricular dilatation disease (PDD). We successfully isolated and characterized ABV from the brains of 8 birds with confirmed PDD. One isolate was passed 6 times in duck embryo fibroblasts, and the infected cells were then injected intramuscularly into 2 healthy Patagonian conures (*Cyanoliseus patagonis)*. Clinical PDD developed in both birds by 66 days postinfection. PDD was confirmed by necropsy and histopathologic examination. Reverse transcription–PCR showed that the inoculated ABV was in the brains of the 2 infected birds. A control bird that received uninfected tissue culture cells remained healthy until it was euthanized at 77 days. Necropsy and histopathologic examinations showed no abnormalities; PCR did not indicate ABV in its brain tissues.

Proventricular dilatation disease (PDD) is a progressive, invariably fatal neurologic disease that has been reported for >50 species of psittacine birds as well as many other bird species (*1*). It is considered a serious disease because many of these birds are highly endangered, and several affected species depend on captive breeding for their survival. The clinical signs of PDD vary and may be predominately neurologic (weakness, ataxia, proprioceptive deficits, seizures, blindness), gastrointestinal (weight loss, passage of undigested food, regurgitation, delayed crop emptying), or a combination thereof (*2*). The gastrointestinal signs, especially proventricular dilatation, are secondary to pseudoobstruction brought about by damage to the enteric nervous system. PDD is characterized by severe lymphoplasmacytic inflammation in peripheral, central, and autonomic nervous tissues (*3*–*5*). Definitive diagnosis of PDD requires demonstration of lymphoplasmacytic ganglioneuritis in the intestinal tract.

Recently, 2 independent groups of investigators identified a new member of the family *Bornaviridae*, named avian bornavirus (ABV), in parrots with histopathologically confirmed PDD. Honkavuori et al. used unbiased high-throughput sequencing to identify the virus in several parrots with histopathologically confirmed PDD (*6*). Quantitative PCR confirmed the presence of the virus in brain, proventriculus, and adrenal gland in 3 birds with PDD but not in 4 unaffected birds. Kistler (*7*) used a panviral microarray to identify a bornavirus hybridization signature in 5 of 8 birds with PDD and 0 of 8 controls. These investigators used ultra high-throughput sequencing combined with conventional PCR-based cloning to recover a complete viral genome sequence. Before this discovery, the family *Bornaviridae* contained only 1 species, *Borna disease virus* (BDV). BDV causes a neurologic syndrome, Borna disease, which is restricted to central Europe, where it is found primarily in horses and sheep. The virus infects neurons and astrocytes, and the resulting disease appears to be mediated by an immunopathologic response of the host to the virus.

BDV can be grown in mammalian cell culture, where it causes a noncytolytic persistent infection. Borna disease appears as a sporadic infection affecting small numbers of animals each year. Its epidemiology is unclear, but it may be carried by certain species of shrews (*8*). BDV has also been detected in the feces of wild birds and in captive ostriches, but the epidemiologic significance of this observa-

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DOI: 10.3201/eid1603.091257

tion is unclear (*9*,*10*). Studies undertaken in this laboratory have demonstrated some histopathologic similarities, in particular in the selective destruction of cerebellar Purkinje cells, between ABV and BDV infections of the brains of birds and mammals, respectively (*11*).

Seven ABV genotypes have been identified based on partial genome sequencing (*12*,*13*). In general, these ABV strains show only ≈65% sequence identity with BDV. Nevertheless, the overall structure of the bornaviral genome is well conserved (*6*,*7*). Thus, the number and order of genes is unchanged, as is the structure of transcription initiation and termination sites. Recently, Rinder et al. (*14*) have shown that the region between the N and X gene in ABV is shorter than that in BDV. ABV apparently lacks a 22-nt fragment that serves a regulatory function for the genes coding for viral proteins X and P.

Although these discoveries suggest that ABV is a plausible cause of PDD, as described in Koch's postulates, proof of a causal relationship requires isolation of the agent from infected birds; its propagation in culture; and, after reintroduction of the isolate into a susceptible host, manifestation of the disease (*15*). We describe the isolation and culture of ABV from the brains of 8 psittacine birds with histopathologically confirmed PDD. After 6 passages, 1 of the cultured isolates was intramuscularly injected into 2 healthy Patagonian conures (*Cyanoliseus patagonis*). Typical PDD subsequently developed in each bird, and the inoculated virus was found in the brain.

Materials and Methods

Parrots

From independent sources we obtained 8 parrots that had clinical signs of PDD, were clinically judged to be in the late stages of the disease, and were euthanized for humane reasons. The 8 birds were 1 green-winged macaw (*Ara chloroptera*), 1 scarlet macaw (*A. macao*), 2 blue and yellow macaws (*A. ararauna*), 2 yellow-collared macaws (*Primolius auricollis*), 1 African gray parrot (*Psittacus erithracus*), and 1 umbrella cockatoo (*Cacatua alba*). Four parrots with conditions not related to PDD and euthanized for humane reasons were also included in the study as negative controls.

Necropsy

Immediately after euthanasia, complete necropsies were performed on all birds. Tissue samples from brain, spinal cord, peripheral nerves, lungs, heart, liver, spleen, pancreas, adrenal glands, kidneys, crop, proventriculus, ventriculus, intestine, and cloaca were placed in 10% buffered formalin for histopathologic examination. Tissue sections were stained with hematoxylin and eosin to confirm the clinical diagnosis. Half of each brain was retained for virus isolation, Western blot, and reverse transcription–PCR (RT-PCR).

Tissue Culture

Specific pathogen–free duck eggs were obtained from the US Department of Agriculture Avian Disease Laboratory (East Lansing, MI, USA). Embryos 9–10 days old were harvested, macerated, and cultured. Primary duck embryonic fibroblasts (DEFs) were used for virus isolation and propagation. DEFs were maintained in Leibowitz L15–McCoy 5A medium (LM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5% bovine calf serum (Sigma-Aldrich) and 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2. DEFs were seeded to confluency 24 h before inoculation. DEFs not inoculated with tissue homogenate were maintained in parallel throughout the experiment.

Virus Isolation and Culture

Brain tissue was harvested immediately after euthanasia. Sections of the cerebrum and cerebellum were homogenized, minced, and then passed through an 18-gauge needle in LM complete medium. In instances where immediate culture inoculation was not possible, the brain tissue was frozen at –80°C within minutes of being harvested and was thawed in a 37°C water bath immediately before inoculation. One milliliter of the brain suspension was used to inject previously plated DEF monolayers that were then incubated for 24 h. The injected DEF cultures were then washed once with phosphate-buffered saline (PBS), replaced with fresh LM medium supplemented with 2% fetal calf serum, and incubated for 5–7 days. Infected DEFs were trypsinized and cocultivated with freshly plated DEFs. This procedure was repeated for a minimum of 3 passages.

Western Blot Analysis

Infected DEFs passaged a minimum of 3 times were used for Western blot analyses. Samples from infected DEFs were collected by trypsinization and pelleted by centrifugation, and pellets resuspended in PBS were sonicated on ice at 50% intensity for 5 min (Sonifier 250; Branson Ultrasonics Corp, Danbury, CT, USA); 50% intensity). Western blotting was performed as described by Towbin et al. (*16*) by using 10% polyacrylamide gels and a Mini-Protean II gel electrophoresis apparatus (Bio-Rad, Hercules, CA, USA). The tissue culture preparations were diluted in sample loading buffer containing β-mercaptoethanol at a ratio of 1:1 and heated to 95°C for 5 min before being loaded (30 μg/slot). A prestained sodium dodecyl sulfate–polyacrylamide gel electrophoresis standard covering the 6.5- to 200-kDa range was used for molecular weight estimation (Bio-Rad).

The size-fractionated antigen preparations were transferred to Immobilon polyvinylidene fluoride transfer mem-

branes (Millipore, Bedford, MA, USA) as described by Towbin et al. (*16*). Transfer efficiency was indicated by the presence of prestained bands on the membranes. After transfer, the membranes were incubated for 2 h in PBS, 0.05% Tween-20, 3% skimmed milk (PBST blocking buffer), then with histopathologically confirmed PDD-positive parrot serum diluted 1:5,000 in PBST blocking buffer for 2 h and with horseradish peroxidase–conjugated goat anti-macaw immunoglobulin Y (Bethyl Inc., Montgomery, TX, USA) diluted 1:10,000 in PBST blocking buffer for 1 h. Membranes were washed with PBST after each step, and all steps were performed at room temperature under constant shaking. Finally, the membranes were incubated for 30 min in Sigma-Fast 3,3′-diaminobenzidine developing substrate (Sigma-Aldrich) and then rinsed in distilled water. The serum from a confirmed PDD-positive parrot used in this experiment has been shown to contain antibodies specific for the 38-kDa ABV N-protein by its reaction with 2 preparations of recombinant protein prepared in *Escherichia coli* and in mammalian cell systems (*11*).

Indirect Immunofluorescent Assay

Infected DEF were washed 2 times for 5 min each in 0.02M PBS, fixed for 10 min in 2% paraformaldehyde in 0.02 M PBS, and washed 2 times for 5 min each in 0.02 M PBS. Cells were permeabilized in 1% Triton X-100/0.02 M PBS for 10 min and washed 3 times for 5 min each in 0.3% Tween/0.02 M PBS. Blocking was performed for 2 h in 5% dried milk/0.3% Tween/0.02 M PBS. The cells were incubated in a humidified chamber for 30 min at 37°C with the primary antibody (serum from a parrot with histopathologically confirmed PDD) at a 1:500 dilution in 1% dried milk/0.3% Tween/0.02 M PBS. Cells were washed 3 times for 5 min each in 0.03% Tween/0.02 M PBS. The cultures were then incubated in a humidified chamber for 30 min at 37°C with the secondary antibody (horseradish peroxidase– or fluorescein isothiocyanate–conjugated goat anti-macaw immunoglobulin G; Bethyl Inc.) at a 1:500 dilution in 1% dried milk/0.3% Tween/0.02M PBS. Cells were washed 3 times for 5 min each in 0.03% Tween/0.02M PBS and then rinsed in distilled water and mounted with ProLong antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA).

RT-PCR

Total RNA was isolated from collected brain tissue and passaged DEF by using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). First-strand cDNA was generated by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), with 1μg RNA and random primers. PCR for ABV N-protein was performed by using 1–2 μL cDNA and forward (5F: 5′-GCGGTAACAACCAACCAGCAA3-′) and reverse (1212R: 5′-GTTCATTAGTTTGCRAATCCRGTTA3-′) primers, which were developed using GenBank submissions NC_001607.1, FJ169441.1, and FJ169440.1 for reference. Amplification conditions were as follows: 1 cycle at 94°C for 2 min; 35 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 80 sec; final extension at 72°C for 7 min. PCR products were cloned in TOPO-TA vector (Invitrogen), and individual clones were sequenced after transformation into *E. coli*. DNA sequencing reactions were performed by using the ABI BigDye Terminator Cycle Sequencing Kit, and sequences were generated with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences were assembled and aligned by using Geneious Pro 4.6.2 software (www.geneious.com). Isolates were assigned to previously defined ABV groups by comparing a 397-nt region to sequences representing avian bornaviruses 1–5 (GenBank accession nos. FJ002329, FJ603688, FJ002328, FJ603687, FJ002335). Evolutionary distances were computed by using a Kimura 2-parameter model with MEGA4 software (www.megasoftware.net).

Experimental Infections

Experimental infections were performed under Animal Use Protocol no. 2009–033B approved by the Texas A&M University Institutional Animal Care and Use Committee. Three adult Patagonian conures were shown to be seronegative by Western blotting and to be ABV negative by fecal PCR. All 3 birds were known to be chronic carriers of psittacine herpes virus; 1 had a cloacal papilloma, but all were otherwise in good health. Psittacine herpes virus has never been implicated in PDD. Two birds were placed in isolation and inoculated by intramuscular injection with infected DEFs containing 8×10^4 focus-forming units (*17*) of an ABV4 (M24) originally isolated from a yellow-collared macaw. A large batch of the M24 strain was grown for 5 days on passage 6, and 500-μL aliquots of this batch were frozen at –80°C in freezing medium. Two of the 500-μL aliquots were grown for 5 days, and immunohistostaining (by using the immunofluorescent antibody [IFA] assay described, substituting the fluorescein isothiocyanate–labeled antibodies with horseradish peroxidase–labeled antibodies) was used to visualize and quantify the focus-forming units.

Results

Isolation and Culture of Avian Bornavirus Isolates

Cytopathic effects were not observed in any of the 12 DEF cultures inoculated with brain tissue harvested from birds with or without clinical signs of PDD. Western blot analyses showed a pronounced ABV N-protein band in extracts of 8 of the 12 cultures. Only DEF cultures inoculated with samples from parrots displaying histopathologically confirmed PDD were positive by Western blotting (Figure 1). ABV N-protein was not detectable in the 4 cultures injected

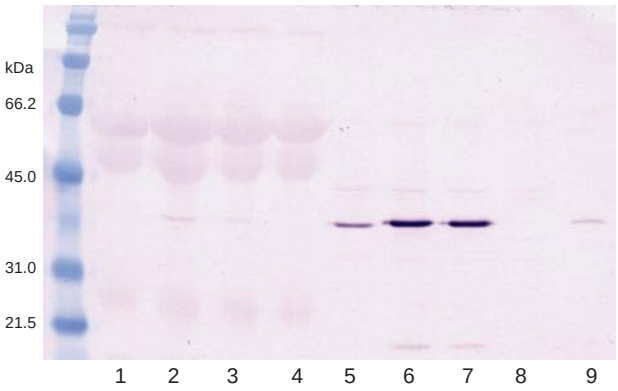

Figure 1. Western blot of infected duck embryonic fibroblasts (DEFs) showing avian bornavirus N-protein during culture. Lanes 1–4 are supernatant fluids. Lane I is from an African gray parrot (AG5). Lanes 2 and 3 are from a yellow-collared macaw (M24). Lane 4 is from uninfected DEFs. Lanes 5–8 are sonicated cell extracts. Lane 5 from AG5; 6 and 7 from M24; and Lane 8 from uninfected DEFs. Lane 9 is an infected brain control. The virus is strongly cell associated.

with brain tissue from birds with no histologic evidence of PDD. IFA of infected DEFs also demonstrated ABV N-protein within cells. Foci of antigen-positive cells were apparent 3 days after culture inoculation. Many cells showed both nuclear and diffuse cytoplasmic staining. Other cells showed the characteristic punctate nuclear staining of infected cultures (Figure 2). No positive immunofluorescence was observed in uninfected DEFs or in DEFs inoculated with brain tissue from negative control birds.

Characterization of Avian Bornavirus Isolates

RNA was isolated from the brain tissues and infected DEF cultures of 8 parrots with PDD and 4 parrots that were PDD negative. A 397-bp region of the ABV N-gene was amplified from all 8 PDD brain and tissue culture samples but not those from the 4 negative parrots. The amplicons were cloned and their sequences compared with previously described ABV groups (*7*). One isolate, M25, was most closely related to ABV group 1, whereas the other 7 ABV isolates were most closely related to ABV group 4 (Table). Pairwise comparisons among the group 4 isolates ranged from 94.2% to 99.7% nucleotide identity. When 2–3 complete N-protein gene sequences (1,143 nt) originating from any bird were compared, nucleotide sequence identity ranged from 99.2% to 100% (data not shown).

Experimental Infections

Two Patagonian conures were challenged with ABV4, strain M24. They were tested by fecal PCR before challenge and at 33, 43, 60, and 62 days postchallenge. Both conures were seronegative by Western blotting before challenge but seropositive for antibodies to the 38-kDa N-protein on day 33 and thereafter. Fecal PCR testing showed that both birds were negative on days 33 and 43. One bird was weakly positive on day 60, but both were strongly positive on day 62. One inoculated bird died on day 66. Necropsy showed a dilated proventriculus and gross lesions characteristic of PDD. Subsequent histopathologic examination confirmed that the bird had a lymphoplasmacytic ganglioneuritis typical of PDD in the crop, proventriculus, gizzard, and intestine (Figure 3). This gangloneuritis included mild to severe infiltration of lymphocytes and a few plasma cells in the serosa, subserosal nerves, and ganglia. The bird also had adrenalitis, encephalitis, and neuritis, as well as a myocarditis. The heart showed a lymphocytic infiltration of the

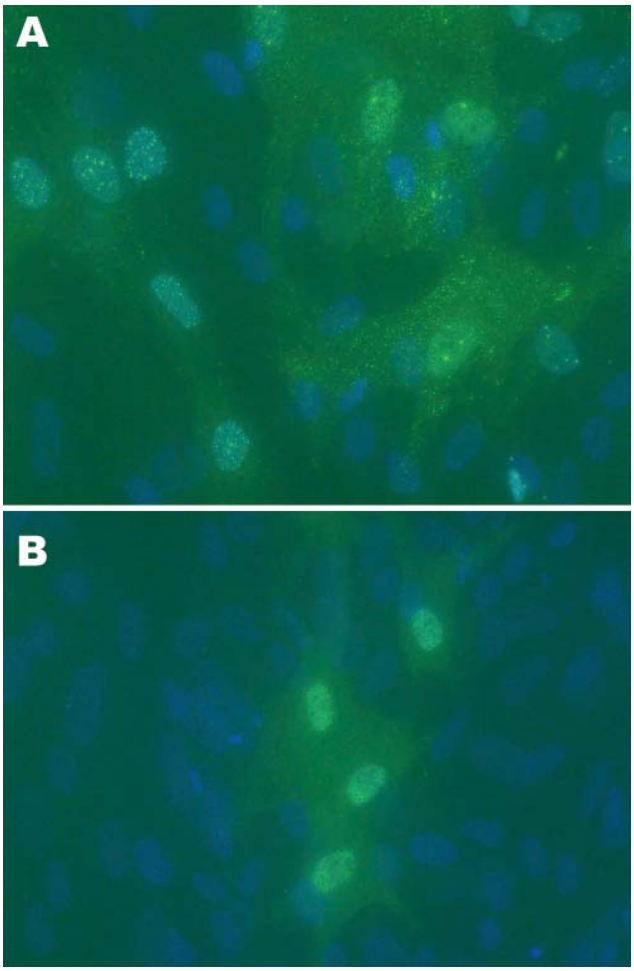

Figure 2. A) Avian bornavirus (ABV)–infected duck embryonic fibroblast (DEF) cell culture 6 days after injection with hindbrain tissues from an African gray parrot with confirmed proventricular dilatation disease (AG5) and staining by an indirect immunofluorescence assay for ABV N-protein. Speckled immunofluorescence is typical of bornavirus infection. Original magnification ×40. B) DEFs 3 days after injection with forebrain from a yellow-collared macaw with confirmed proventricular dilatation disease (M24). Nuclear and cytoplasmic fluorescence in DEFs stained by immunofluorescence assay for ABV N-protein. Original magnification ×40.

Table. Percent nucleotide identity between partial N genes in avian bornavirus isolates

Gene	ABV Type 1a*	M25	M20	AG5	M15	M10	M14	M24	06	ABV Type 4b†
ABV Type 1a	100.0									
M25	89.4	100.0								
M20	80.9	82.0	100.0							
AG5	80.9	82.0	99.7	100.0						
M15	80.9	82.0	99.9	99.7	100.0					
M10	80.9	82.0	99.6	99.5	99.7	100.0				
M14	81.1	81.7	99.6	99.6	99.7	99.6	100.0			
M24	81.1	81.7	95.0	94.8	94.7	94.5	94.5	100.0		
06	81.4	81.8	94.5	94.5	94.4	94.3	94.2	99.3	100.0	
ABV Type 4b	80.6	81.4	94.0	94.7	94.7	94.7	95.0	92.7	94.5	100.0

*ABV genotype 1 accession no. FJ002329.
†ABV genotype 4 accession no. FJ603687.

epicardial ganglia as well as in and around Purkinje fibers. Thus, the brain and spinal cord showed multifocal perivascular cuffing and gliosis (Figure 4). The adrenal medulla was infiltrated with lymphocytes and plasma cells.

The second inoculated bird was examined on day 66 and was found to be emaciated and had clinical signs consistent with PDD. It was euthanized for humane reasons. This bird also had gross and histopathologic lesions characteristic of PDD, essentially identical to those described above. The brains of both conures were subjected to PCR for ABV N-protein as described above. Results for both were positive (Figure 5). Sequence analysis of the PCR products confirmed that bird brains contained ABV4 identical to the M24 challenge strain. Brain homogenates from these 2 birds were also cultured on DEFs, and a strong positive PCR signal was obtained at day 16 of culture.

The third conure in this study received uninfected DEFs by both intramuscular and oral routes as described for experimentally infected birds. This bird was housed in an aviary separate from the isolation facilities of the infected birds. It was in apparent good health when euthanized on day 77. Necropsy of the bird conducted, including histopathologic examination of its tissues, and PCR was performed on 4 regions of its brain. No evidence of PDD was seen during necropsy or histopathologic examination, and all 4 brain samples were negative for ABV nucleic acid.

Discussion

Although it has long been proposed that a viral pathogen was responsible for PDD, past attempts to identify a causal agent through inoculation of chick embryos and a variety of tissue cultures were unsuccessful. Because no cytopathic effects were detected in the DEF cultures after several passages, prior attempts to grow the agent may have been successful but had not been recognized because of lack of immunologic or PCR detection tools. We were able to isolate and propagate ABV from all studied birds with clinical PDD. IFA of infected DEF using this same antiserum showed the punctate nuclear staining that is typical of cells infected with bornaviruses and appears to be the result of the formation of N-and P-protein complexes known as Joest-Degen inclusion bodies (*18*–*20*). It is noteworthy that we were unable to grow ABV in primary chicken embryo fibroblasts handled the same as the DEFs. Rinder et al. reported successful propagation of ABV in the chicken LMH hepatoma cell line (*14*). However, they noted slow growth and only a few positive cells compared with propagation in the quail fibroblast cell line CEC32 and the quail skeletal muscle cell line QM7. Thus ABV appears to have constraints in host cell range. PDD in chickens has not been reported. Rinder et al., like ourselves (P. Gray et al., unpub. data), were unable to grow ABV in cell lines of mammalian origin, such as Vero cells or MDCK cells in which BDV grows routinely. This research finding suggests that ABV may be unable to infect mammals.

Bornavirus has a nonsegmented negative strand genome. It encodes at least 6 proteins: N, X, P, M, G ,and L. The N or nucleoprotein interacts with the viral RNA and accumulates in the nucleus during the life cycle of the virus (*21*,*22*). The nucleoprotein of BDV exists in 2 isoforms of 40 and 38 kDa (*23*,*24*). P40 is primarily nuclear, and

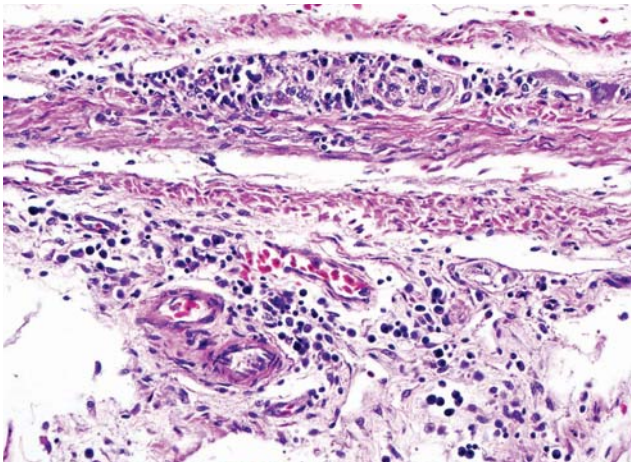

Figure 3. Proventriculus wall from conure PG8 showing characteristic lymphoplasmacytic infiltration of the subserosal enteric ganglia as well as infiltration of submucosa. This bird had been inoculated 55 days earlier with avian bornavirus, genotype 4. Original magnification ×325.

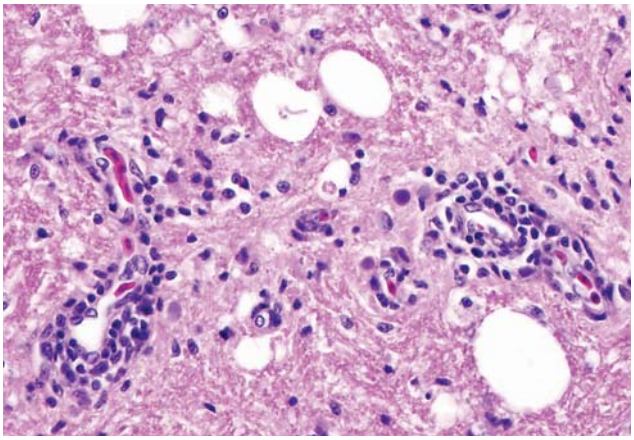

Figure 4. Lymphoplasmacytic encephalitis with multifocal perivascular cuffing in the cerebrum of conure PG8 inoculated 55 days earlier with avian bornavirus genotype 4. Original magnification ×725.

P38 is primarily cytoplasmic. Both isoforms can bind to the viral phosphoprotein. The immunofluorescent staining pattern observed with ABV-infected DEFs, which showed a punctate nuclear staining combined with a more diffuse cytoplasmic staining, is thus compatible with the known properties of the BDV nucleoprotein. Of 8 isolates reported here, 7 were of genotype 4 and 1 was of genotype 1. This finding may suggest that genotype 4 is a more pathogenic type associated with disease, or it may simply be the predominant strain circulating in Texas.

Rinder et al. (*14*) reported on 6 isolates from Germany, 4 of which were genotype 4 and the others were genotype 2. This finding supports the suggestion that genotype 4 may be predominant worldwide and possibly more virulent than other genotypes. The experimental infection of 2 Patagonian conures with cultured virus that resulted in clinical PDD 66 days postinfection fulfills Koch's postulates. PCR and sequencing of the amplified product demonstrated large amounts of ABV4 in the brains of the challenged birds. The birds did seroconvert for anti-N antibodies at 33 days, whereas fecal shedding was not detected until days 60–62. This finding is in contrast to observations on naturally infected birds in which fecal shedding may precede seroconversion by many months (*25*). ABV RNA was detected by RT-PCR after a minimum of 3 passages in DEF primary cell culture subsequent to inoculation with brain tissue from all 8 necropsy-confirmed PDD-positive birds. PCR detection in the brain tissue and ready isolation of the virus from freshly harvested brain tissue are compatible with the concept that PDD originates as a viral encephalitis (*11*).

Gancz et al. (*26*) have induced PDD in cockatiels (*Nymphicus hollandicus*) after inoculation of brain homogenates from PDD-affected, ABV-positive birds. Although the findings of Gancz et al. support our results and are in line with previous findings (*27*), interpretation of their results is difficult because of evidence for an autoimmune component in PDD similar to that which occurs in Guillain Barré syndrome (*28*). We also have detected autoantibodies to myelin basic protein and other nervous system autoantigens in PDD cases, suggesting that in this study, the brain homogenate may have contributed to the abnormalities observed. (*25*). The known pathogenesis of mammalian bornavirus infections fits well with the causative role of ABV in PDD. Both PDD and mammalian Borna disease share many attributes, including a viral encephalitis and polyneuritis with selective destruction of Purkinje cells, lymphocyte infiltration, and dysfunction of the central, peripheral, and autonomic nervous systems (*29*–*31*).

In conclusion, the results reported here together with previous findings confirm unequivocally that the long-sought cause of proventricular dilatation disease is indeed

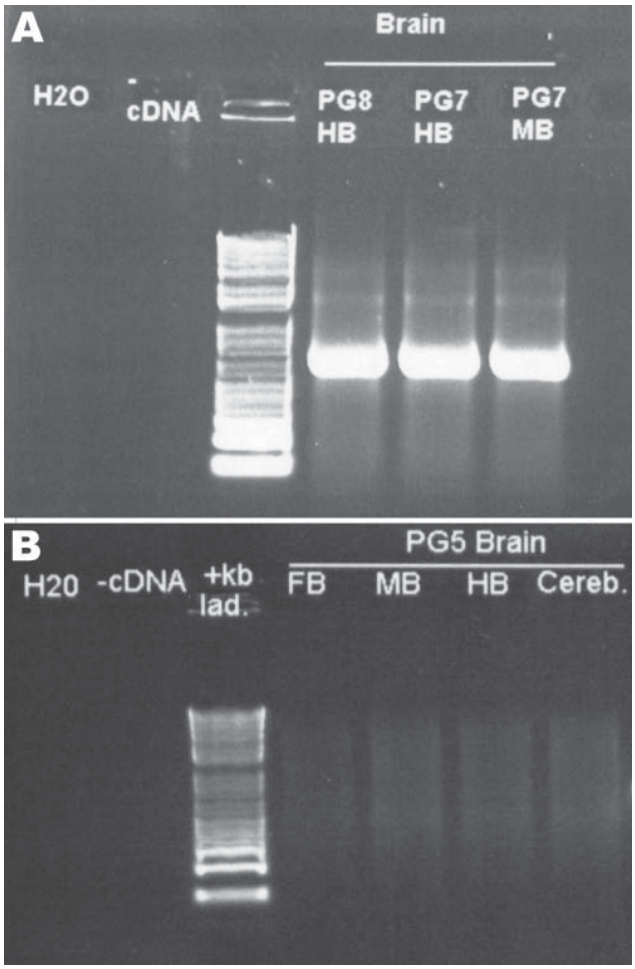

Figure 5. PCR of avian bornavirus N-protein in different areas of the brains of A) 2 Patagonian conures (PG7 and PG8) inoculated 55 days earlier with avian bornavirus–infected duck embryonic fibroblasts and B) control, uninfected bird, PG5. HB, hindbrain; FB, forebrain; MB, midbrain; Cerebr., cerebrum.

avian bornavirus. Investigations into this virus and the complex disease that it causes may provide useful insights into the pathogenesis of mammalian Borna disease. The origin and epidemiology, as well and prevention and treatment, of this infection remain to be elucidated.

This research was supported by the Richard M. Schubot Endowment at Texas A&M University.

Dr Gray is a resident in avian studies and works at the Schubot Exotic Bird Health Center, where she has been conducting research on proventricular dilatation disease.

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Economic Cost Analysis of West Nile Virus Outbreak, Sacramento County, California, USA, 2005

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In 2005, an outbreak of West Nile virus (WNV) disease occurred in Sacramento County, California; 163 human cases were reported. In response to WNV surveillance indicating increased WNV activity, the Sacramento-Yolo Mosquito and Vector Control District conducted an emergency aerial spray. We determined the economic impact of the outbreak, including the vector control event and the medical cost to treat WNV disease. WNV disease in Sacramento County cost ≈$2.28 million for medical treatment and patients' productivity loss for both West Nile fever and West Nile neuroinvasive disease. Vector control cost ≈$701,790, including spray procedures and overtime hours. The total economic impact of WNV was $2.98 million. A cost-benefit analysis indicated that only 15 cases of West Nile neuroinvasive disease would need to be prevented to make the emergency spray cost-effective.

After its introduction into the eastern United States in 1999, West Nile virus (WNV) reached California in 2003 (*1*). In response, the state enhanced mosquito management programs to reduce vector populations and virus transmission (*2*). By late summer 2005, WNV disease was epidemic in Sacramento County, with more cases reported in Sacramento County than in any other county in the nation that year (*3*). The Sacramento-Yolo Mosquito and Vector Control District (SYMVCD) responded by conducting emergency aerial spraying over the city of Sacramento and surrounding areas to reduce mosquito populations.

Effective management of infection rates, illness, and death from mosquito-borne pathogens such as WNV requires reduced contact between humans and infected mosquitoes (*4*). No effective treatment exists for WNV; prevention of disease relies on management of mosquitoes through various control tactics. Elnaiem et al. (*5*) and Carney et al. (*6*) examined the efficacy of the 2005 emergency aerial spray in Sacramento County, which used pyrethrins as the active ingredients to control adult mosquitoes. In both studies, an unsprayed area within the county was used as the control. Elnaiem et al. showed a total decrease in WNV-competent vector mosquitoes, *Culex pipiens* and *Cx. tarsalis*, of 57.5%, compared with the prespray population in the treated area (*5*). They also observed a decrease in WNV infection rates in mosquitoes to 3.9/1,000 for trapped females in the treated areas, compared with 6.7/1,000 in the untreated areas (*5*). Carney et al. used illness onset dates and residential locations for 152 of the 163 WNV disease cases reported in humans in 2005 to determine the efficacy of the spray event (*6*). Their results showed no incident human cases in the treated area after the spray event, compared with 18 cases in the untreated area. Consequently, the emergency aerial spray seemed to effectively reduce both mosquito populations (*5*) and human WNV cases (*6*).

WNV infection can be asymptomatic or symptomatic in humans, with a 4:1 ratio (*7*,*8*). The disease can be mild, resulting in influenza-like symptoms (as in West Nile fever [WNF]), or severe, affecting the central nervous system symptom (as in West Nile neuroinvasive disease [WNND]) (*7*). Many WNF cases are not reported because they are not recognized as WNF; symptoms can resemble a cold or mild influenza-like illness, for which medical care is not sought, or is underdiagnosed because the additional cost of testing would not provide alternative direction to effective palliative medical care (*7*,*9*).

Zohrabian et al. (*10*) estimated the economic impact of the WNV disease outbreak in 2002 in Louisiana, which resulted in 24 deaths. They included costs of inpatient and outpatient medical care, productivity loss, the state's public

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DOI: 10.3201/eid1603.090667

health department, and vector control. Total epidemic costs were ≈$20.14 million for the 329 cases, including $9.2 million for mosquito control and public health agency costs. Zohrabian et al. (*11*) used the economic data from their 2004 study to determine the cost-effectiveness of the initiation of a potential WNV vaccination and found that the cost of vaccination would not offset the costs in medical care.

Several studies have demonstrated the efficacy of mosquito management in response to WNV, but only the study by Carney et al. (*6*) suggested a reduction in human WNV cases associated with aerial adult-mosquito control. We estimated the economic cost of the 2005 WNV disease outbreak in Sacramento County, California, and evaluated the reduction in WNV disease necessary to offset the cost of emergency vector control. Economic costs for patients' productivity loss and for treatment of disease symptoms, as well as for emergency vector control conducted in response to the outbreak were also investigated.

Methods

Medical Costs

We estimated costs for the total number of Sacramento County WNV cases in 2005. Different costs were associated with WNF and the more severe WNND. The Centers for Disease Control and Prevention (CDC) summarizes the reported number of WNV cases for each state, including patient's age, sex, date of onset, case reporting date, county of residence, diagnosis (WNF or WNND), and outcome (e.g., fatal). According to the CDC database for 2005, a total of 935 human WNV cases were reported in California, including 163 cases from Sacramento County (*3*). A total of 117 (71.8%) were diagnosed as WNF and 46 (28.2%) as WNND; 1 (0.6%) case was fatal. Forty-six (28.2%) patients were ≥60 years of age, and 2 (1.2%) were <18 years of age.

For WNND, we calculated costs using similar methods as and specific data from Zohrabian et al. (*10*). Costs of inpatient and outpatient care, lost productivity, and miscellaneous expenses were summed to estimate the total cost of an individual WNND case. Costs for WNF, including average price for a physician visit, CDC-approved diagnostic testing, and productivity loss during symptomatic WNV disease, were summed to estimate the total cost of an individual WNF case.

WNND

We obtained inpatient costs for WNND using the 2005 hospital patient discharge database from California's Office of Statewide Health Planning and Development (OSHPD) (J. Teague and J. Morgan, pers. comm.). This database included patients with a WNV-related diagnosis who were admitted to hospitals within Sacramento County's ZIP codes. It also included average inpatient hospital charge per stay and average length of stay for the different WNV diagnosis codes (Table 1). Cost data were available for 16 of the 27 WNND cases reported by Sacramento County hospitals in 2005 (some hospitals do not report cost data). Charges were averaged for each diagnosis code, and the average charge was determined for WNND (no hospital cases were reported for WNF). The average charge was then converted to the true economic cost by using the average Sacramento County hospital cost-to-charge ratio (CCR). Individual hospitals' CCRs were obtained from California's Department of Industrial Regulations (*12*), and the average was based on the number of cases reported at each hospital in the county, also obtained from OSHPD. The resulting inpatient cost was extrapolated to all WNND cases in Sacramento County for the total economic impact.

We estimated outpatient costs for WNND using the 2002 outpatient costs determined by Zohrabian et al. (*10*) and updated to 2005 using data from the Consumer Price Index (CPI) for the western United States, obtained from the US Department of Labor, Bureau of Labor Statistics (*13*–*15*). Zohrabian et al. used hospital cost data reported from 119 patients and phone surveys of 139 patients to determine related treatment costs for WNV disease symptoms. CPI data included the percentage increase for medical care services for 2002–2003, 2003–2004, and 2004–2005. These increases were applied to the service categories orig-

Table 1. WNF diagnosis codes and cases, Sacramento County, California, 2005*

Diagnosis code	Diagnosis	No. hospitalized case-patients reported†	Classification	Cases with cost data‡	Average ALOS§ (range¶)
66.40	WNF, unspecified	0	WNF	0	
66.41	WNF with encephalitis	17	Severe (WNND)	14	16 (12–36)
66.42	WNF with other neurologic manifestation	8	Severe (WNND)	2	13 (10–14)
66.49	WNF with other complications	2	Severe (WNND)	0	21 (13–29)
WNND totals or averages		27		16	15 (10–36)

*WNF, West Nile fever; ALOS, average length of stay (J. Teague and J. Morgan, pers. comm.); WNND, West Nile neuroinvasive disease.
†Cases in 2005 obtained from the Patient Discharge Data from California's Office of Statewide Health Planning and Development (J. Teague and J. Morgan, pers. comm.).
‡Cases that included cost data from the Patient Discharge Database and incorporated into the study.
§Average length of stay, in days (J. Teague and J. Morgan, pers. comm.).
¶Not the true ALOS range, determined from data for each diagnosis code.

inated by Zohrabian et al.: hospital treatment, physician visits, outpatient physical therapy, occupational therapy, and speech therapy. Percentages of total patients for whom the service applied were determined by using information from Zohrabian et al. for each outpatient category; these percentages were then applied to the Sacramento County WWND cases for the costs per patient per category.

Miscellaneous costs included nursing home, transportation, home-health aides, and child care costs accrued during recovery from WNND. Average nursing home costs per day in 2005 were obtained from the Survey of Nursing Home and Home Care Costs (*16*). We calculated the value by averaging the national costs for the daily rate of a private and semiprivate room in 2005. The total associated costs for a nursing home stay was then determined by multiplying this value by the average number of days a WNND patient spent in a nursing home (96 days) (*10*). We applied this cost to 3.6% of the WNND patients and rounded it to the nearest whole number of patients. Transportation, home-health aides, child care, and other home-help costs were calculated by using the cost values determined in Zohrabian et al., updated to 2005 by using the CPIs mentioned previously (*13*–*15*). We applied the resulting transportation cost to all WNND cases, and applied costs for home-help aides to 14.4% of the 2005 WNND cases and rounded to the nearest whole number of patients.

We assumed that productivity loss differentially affected persons in 2 age groups: ≥60 years and <60 years. Productivity loss was also calculated for nonprofessional caretakers of WNND patients. We determined the cost for a day of work missed by an average Sacramento adult citizen using the mean annual earnings for full-time workers in 2005 (*17*). Annual income was divided by 250 work days per year. The resulting value was the cost for a day of work missed by persons <60 years of age. We calculated the cost for a nonwork day missed using Productivity Loss Tables from 2000 (*18*) and updated to 2005 dollars using the US Department of Labor, Bureau of Labor Statistics, annual earnings (*17*). The percentage increase from 2000 to 2005 was applied to the Productivity Loss Tables' value for a nonwork day loss. The resulting value for a nonwork day missed also was used for productivity loss for persons ≥60 years of age who had WNND. We conservatively assumed an average of 50 work days missed (*10*) and 10 nonwork days missed (1 weekend day per week).Thus, total productivity loss was 60 days. For caretakers of WNND patients, productivity loss was assumed to be 25 days, and the associated cost was the value of a nonwork day missed (*10*). The cost attributed to productivity loss is an estimate; true monetary value for pain and distress and the productivity loss associated with chronic WNND are uncertain.

WNF

Assumed costs for treating WNF were those of a physician visit, a diagnostic test, and productivity loss during symptomatic WNF. We obtained the average costs for a physician visit for a diagnosis or treatment in the western United States from 2004 data (*19*) and updated to 2005, using the CPI (*15*) as discussed above.

The CDC-approved diagnostic test for human WNV is an immunoglobulin (Ig)M and IgG ELISA for either serum or cerebrospinal fluid (*7*). According to CDC, an additional test is needed to indicate a false-positive result; however, our analysis assumed only costs for the initial diagnostic test. We obtained this value by contacting 4 laboratories suggested by the California Department of Public Health (C. Jean, pers. comm.) (ARUP Laboratories, Salt Lake City, UT, USA; Focus Diagnostics Inc., Cypress, CA, USA; Quest Diagnostics Inc., Madison, NJ, USA; and Specialty Laboratories, Valencia, CA, USA); the costs obtained were then averaged. Productivity loss for a missed day of work and a missed day of nonwork were calculated by using the methods detailed previously. We assumed 5 workdays missed because of WNF for persons <60 years of age and 5 nonwork days missed for persons ≥60 years of age.

Cost of Mosquito Vector Control

We obtained cost information for the 2005 emergency mosquito control aerial spray from SYMVCD. It included aerial ultra-low–volume adulticiding over 2 areas in Sacramento County comprising ≈477 km^2 (*6*). Aerial spraying was conducted on 6 nights in early and mid-August (*5*). The event costs incorporated overtime hours for SYMVCD employees for August 2005. We calculated total overtime hours spent on the emergency spray using the difference between paid overtime hours for August 2005 and August 2004. Overtime hours for August 2005 were assumed to be additional hours to SYMVCD's usual vector control program, including hours for additional prespray and postspray application mosquito trapping, plane preparation time, and preparation time for completing the spraying. These hours included time spent on other spray events and vector control procedures not directly involved in the emergency spray. However, our study incorporated total overtime hours for August to ensure conservatism. Total cost for the emergency spray also included outsource contracts (e.g., plane rental, pilot hours) and the insecticide used.

Results

Medical Costs for WNND

A total of 46 WNND cases occurred in Sacramento County in 2005. Costs were ≈$33,143 per inpatient and ≈$6,317 per outpatient for all treatments (Table 2). Cost

Table 2. Estimated inpatient and outpatient economic costs of WNND cases, Sacramento County, California, 2005*

Item	Cost per case†	No. cases to which cost applies‡	% Cases to which cost applies§	Total cost for all cases	Total cost if treatment/service were used in all cases
Inpatient treatment costs	$33,143	46	100	$1,524,570	$1,524,570
Outpatient costs	Cost per case¶				
Outpatient hospital treatment	$333	17	36	$5,668	$15,337
Physician visits	$450	46	100	$20,708	$20,708
Outpatient physical therapy	$909	46	100	$41,810	$41,810
Occupational therapy	$4,037	3	7	$12,111	$185,699
Speech therapy	$588	1	1	$588	$27,032
Total				$80,885	$290,586
Nursing home costs	Cost#				
Nursing home stay**	$190	2	4	$36,195	$36,195
Transportation	$65	46	100	$2,977	$2,977
Home health aides, babysitters, etc.	$1,569	7	14	$10,983	$505,211
Total				$50,154	$544,383
Total for all WNND				$2,140,409	$2,844,339

*WNND, West Nile neuroinvasive disease; BLS, Bureau of Labor Statistics of the US Department of Labor.
†Estimated by using 2005 data from California's Office of Statewide Health Planning and Development (J. Teague and J. Morgan, pers. comm.).
‡WNND cases from the total number of cases reported by the Centers for Disease Control and Prevention (*3*).
§See (*10*).
¶Estimated by using data from Zohrabian et al. (*10*) and updated using data from the US Department of Labor's Bureau of Labor Statistics (BLS) (*13*–*15*).
#Estimated by using data from MetLife Mature Market Institute (*16*), Zohrabian et al. (*10*), and BLS (*13*–*15*).
**Average length of nursing home stay was 96 days.

for each WNND patient estimated to have spent time in a nursing home was ≈$18,097. Productivity loss during symptomatic WNND cost $10,800 per patient <60 years of age and $7,500 per patient ≥60 years of age (Table 3). Total medical costs accrued by all WNND patients was ≈$2,140,409; total costs for all cases (medical cost plus productivity loss) was ≈$2,844,338.

We performed sensitivity analysis for medical treatment of WNND in which we had a range of values using 10,000 iterations. The hospitals' CCRs contributed the largest amount of variance to the total cost (68.5%), followed by the average inpatient cost per WNND patient from the 2005 hospital patient discharge database from OSHPD (J. Teague and J. Morgan, pers. comm.) (31.4%), range $1,910,421–$7,770,354. Results were similar for the cost per WNND inpatient (range $13,201–$140,257) and the total medical cost for treating WNND.

Medical Costs for WNF

A total of 117 WNF cases were reported for Sacramento County in 2005. Treating each WNF patient cost ≈$167 for the diagnostic physician visit and ≈$135 for the diagnostic test. Productivity loss cost ≈$955 for each patient <60 years of age and $625 for each patient ≥60 years of age. The total cost for treating reported WNF cases was ≈$136,839 (Table 4).

Sensitivity analysis for the cost of treating WNF (range $132,008–$144,458) showed that the average cost for the diagnosis test contributed the largest amount of variance to the total cost (84.2%). The cost of a missed day of work for patients <60 years of age was 15.8%.

Emergency Vector Control Spray

The emergency spray comprised 1,157 additional overtime hours in SYMVCD for August 2005. These overtime hours cost ≈$41,790. The emergency spray cost ≈$660,000 (D. Brown, pers. comm.). Therefore, the emergency aerial spray response to the WNV epidemic cost a total of $701,790.

Total Costs and Potential Benefits

Total cost of the 2005 Sacramento County WNV epidemic was ≈$2,979,037. Costs for treating WNND patients alone exceeded costs of emergency vector control by $1,438,619, a ratio of 3:1. This difference suggests that for the benefits of the vector control to outweigh the cost of the epidemic, the spray event would need to prevent only 15 WNND cases.

Table 3. Estimated economic costs of WNND cases due to productivity loss, Sacramento County, California, 2005*

Productivity loss	Value of work day missed†	Value of nonwork day missed‡	No. work days missed	No. nonwork days missed	No. patients <60	No. patients ≥60	% Cases	Total costs for all cases
For patients <60 y	$191	$125	50	10	31		100	$334,800
For patients ≥60 y		$125		60		15	100	$112,500
For caretakers		$125	25		8	4	26	$37,500
Total costs								$484,800

*WNND, West Nile neuroinvasive disease.
†Estimated by using data from BLS (*17*).
‡Estimated by using data from Grosse (*18*) and BLS (*17*).

Table 4. Estimated economic impact for WNF cases (N = 117), Sacramento County, California, 2005*

Item		Cost	No. patients by age, y <60	≥60	Total cost
Physician visit for diagnosis or treatment in the western US, cost per case†		$167	86	31	$19,539
Diagnostic tests, average cost per case‡		$135			
Productivity loss	Per work day missed§	Per nonwork day missed¶	Total individual cost#		
Value of a lost day	$191	$125	$955	$625	$101,505
Total costs for WNF					$136,839

*WNF, West Nile fever; BLS, Bureau of Labor Statistics, US Department of Labor.
†Estimated by using data from Brown and Beauregard (*19*) and BLS (*15*).
‡ELISA immunoglobulin (Ig) G and IgM serum and cerebrospinal fluid. Estimated by using laboratory list prices (ARUP Laboratories, Salt Lake City, UT, USA; Focus Diagnostics Inc., Cypress, CA, USA; Quest Diagnostics Inc., Madison, NJ, USA; Specialty Laboratories, Valencia, CA, USA).
§Estimated by using data from BLS (*17*).
¶Estimated by using data from Grosse (*18*) and BLS (*17*).
#Based on 5 workdays missed per person <60 y and 5 nonwork days missed per person >60 y.

Discussion

Since 1999, when WNV was detected in the United States, several studies have evaluated the efficacy of vector control, especially adulticide treatments. Palmisano et al. (*20*) observed an 86% decrease (compared with a 5-year average) in WNV-vectoring mosquitoes in 2002 resulting from control efforts over a 4-month period in St. Tammany Parish, Louisiana. Simpson (*21*) observed a 64% reduction in WNV-carrying mosquito species measured during emergency aerial sprays in 26 Florida counties during 2004 in response to hurricanes. Carney et al. (*6*) and Elnaiem et al. (*5*) provided evidence of the effectiveness of the 2005 emergency aerial spray as a mosquito control measure in Sacramento County by showing a reduction both in mosquito populations and WNV disease cases in humans.

Carney et al. (*6*) documented 18 total WNV disease cases outside the spray area after the Sacramento County emergency spray and no cases within the spray area, after they adjusted for the maximum incubation period of the virus from infection to onset of symptoms. Of these 18 cases, 13 were diagnosed as WNF and 5 as WNND. Treating these 18 patients cost ≈$241,462. However, given the possibility of unreported or underdiagnosed WNF cases, the spray event may actually have prevented >18 cases (*7*,*9*,*22*,*23*). SYMVCD activities conducted before the emergency period most likely prevented some cases.

Estimating the medical costs of WNV patients and the true number of cases prevented by the emergency spray are uncertain. The estimated dollar amount designated for productivity loss from WNV disease was based on the average annual salary of a Sacramento County citizen in 2005 and an estimated number of work days missed because of the disease. This study does not take into account extreme cases of WNND and total number of days a patient is affected by the disease. Therefore, the actual cost values associated with WNV may be higher.

Our analysis may underestimate the actual cost of the WNV outbreak. Pain and distress are difficult to estimate monetarily but probably are important factors in the comprehensive costs of WNV disease. We also did not include medical costs associated with non-WNV issues, such as mosquito-bite allergenicity or sequelae, which are difficult to quantify but may be substantial (*24*). Additionally, we did not incorporate the benefits to the human population of reducing the nuisance of mosquito bites, irrespective of WNV transmission. In Jefferson County, Texas, the ratio of the cost of the total household benefit to the program cost for mosquito abatement was 1.8, according to a county-wide study on the benefit of mosquito control in reducing the nuisance of mosquito bites (*25*). In addition, the actual number of persons affected with WNF remains unknown because the total number of WNF cases probably was underreported and underdiagnosed (*7*,*9*). Busch et al. (*26*) found 353 infections for each reported case of WNND in North Dakota from blood screening data in 2003 compared with CDC data indicating ≈256 WNV incident infections for each WNND case in the United States.

We did not assess human and ecologic risks associated with the emergency spray. However, previous risk assessments that used exposure scenarios for pyrethroids and pyrethrins that would exceed those of the Sacramento County emergency aerial spray have shown risks substantially below Environmental Protection Agency levels of concern (*27*–*34*).

The total economic impact of the 2005 WNV disease outbreak in Sacramento County was ≈$2.98 million. The total cost of medical treatment for the outbreak was $2.28 million. The actual number of WNV disease cases prevented by the emergency spray is uncertain. However, the offset in cost for the number of cases that may have been prevented can be compared with the costs of the vector control. If only 34 WNF and 14 WNND cases (by using the percentages of each from the diagnoses for Sacramento County in 2005) were prevented by the spray event, ≈$702,809 would have been averted in medical and productivity loss costs, thus offsetting the cost of the emergency spray. Also, the costs of the emergency spray would have been offset by preventing only 15 WNND cases at ≈$706,833.

Acknowledgments

We thank D. Brown, G. Goodman, and P. Macedo for providing information about the vector control costs. We also thank J. Teague and J. Morgan for relaying the hospital cost data to us and E. Geraghty and G. Trochet for their input. We appreciate the assistance of the California Department of Public Health, especially C. Jean, A. Kjemtrup, V. Kramer, and M. Novak for help in data acquisition and review of earlier manuscripts.

The research was supported by Montana State University and the Montana Agricultural Experiment Station.

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Paenibacillus larvae Bacteremia in Injection Drug Users

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Paenibacillus larvae causes American foulbrood in honey bees. We describe *P. larvae* bacteremia in 5 injection drug users who had self-injected honey-prepared methadone proven to contain *P. larvae* spores. That such preparations may be contaminated with spores of this organism is not well known among pharmacists, physicians, and addicts.

As a consequence of needle sharing and repeated parenteral administration of nonsterile material, injection drug users risk becoming ill from a variety of infections, including HIV, hepatitis C, endocarditis, and skin and soft tissue infections (*1*). Febrile episodes in injection drug users are common, yet distinguishing between febrile reactions caused by toxins or impurities in the injected substance and true infections may be difficult (*2*,*3*). Methadone hydrochloride, which is widely used for opioid substitution, can be mixed with viscous substances such as syrup to yield a solution that is not suitable for misuse through self-injection. Methadone syrup is intended to be taken only as an oral medication. Some pharmacies use honey instead of syrup to prepare such a solution.

Paenibacillus larvae is a spore-forming gram-positive microorganism known for its ability to cause American foulbrood, a severe and notifiable disease of honey bees (*Apis mellifera*) (*4*) (Figure). *P. larvae* is endemic to bee colonies worldwide. The organism can be cultured from <10% of honey samples from Germany but from >90% of samples from honeys imported from other countries (*5*). *P. larvae* spores are highly resilient and can survive in honey for years (*6*,*7*). We describe *P. larvae* bacteremia in 5 patients who had a history of intravenous drug abuse and were in a program of opioid substitution that used methadone.

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DOI: 10.3201/eid1603.091457

The Study

All patients sought treatment for fever ranging from 37.8°C to 39.8°C and admitted to continuing to inject illicit drugs or methadone. Information about patient characteristics, clinical signs and symptoms, laboratory and micobiologic investigations, and treatment details are summarized in the Table. *P. larvae* was identified in blood cultures (BacT/ALERT 3D-System; bioMérieux, Marcy l'Etoile, France) of each patient described. The clinical course of *P. larvae* bacteremia was benign in 3 patients, and complications developed in 2 patients. Patient 1 had relapsing disease and spontaneous bacterial peritonitis; patient 4 had pulmonary embolism without definite evidence of septic embolism. Patients 2 and 3 recovered without specific antimicrobial drug treatment; for patients 4 and 5, defervescence and negative follow-up blood cultures were observed after they received treatment with β-lactam agents (imipenem or cefuroxime). The recurrent *P. larvae* infection observed in patient 1 was probably the consequence of repeated injection of contaminated methadone rather than an inadequate response to antimicrobial drug therapy.

In 2 cases, culture of the honey used to prepare methadone or of honey-containing ready-to-use methadone also yielded *P. larvae*. Honey and methadone samples were diluted in sterile phosphate-buffered saline and cultured with or without heat pretreatment (90°C, 10 min) under aerobic and anaerobic conditions at 37°C for 3–4 days by using Columbia blood agar and MYPGD (Mueller-Hinton broth, yeast extract, potassium phosphate, glucose, pyruvate) agar. Colonies from positive blood or honey or methadone cultures with an appropriate macroscopic appearance and gram-stain morphology as well as negative catalase reaction were further identified by PCR amplification and 16S rRNA gene sequencing according to published protocols (*8*). Obtained sequences were analyzed by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Conclusions

We detected *P. larvae* in sterile compartments of 5 patients with clinical and laboratory evidence of infection. Given the fact that all patients were injection drug users, the mode of infection was thought to be intravenous administration of contaminated methadone, resulting in *P. larvae* bacteremia. Our hypothesis is supported by the isolation of *P. larvae* from honey or honey-containing methadone provided to 2 patients.

Recently, several *Paenibacillus* species have been reported to cause bacteremic infections in humans. Among these are *P. thiaminolyticus* (bacteremia in a patient undergoing hemodialysis) (*9*), *P. konsidensis* (bacteremia in a febrile patient with hematemesis) (*10*), *P. alvei* (prosthetic joint infection with bacteremia) (*11*), and *P. polymyxa* (bacteremia in a patient with cerebral infarction) (*12*). Further-

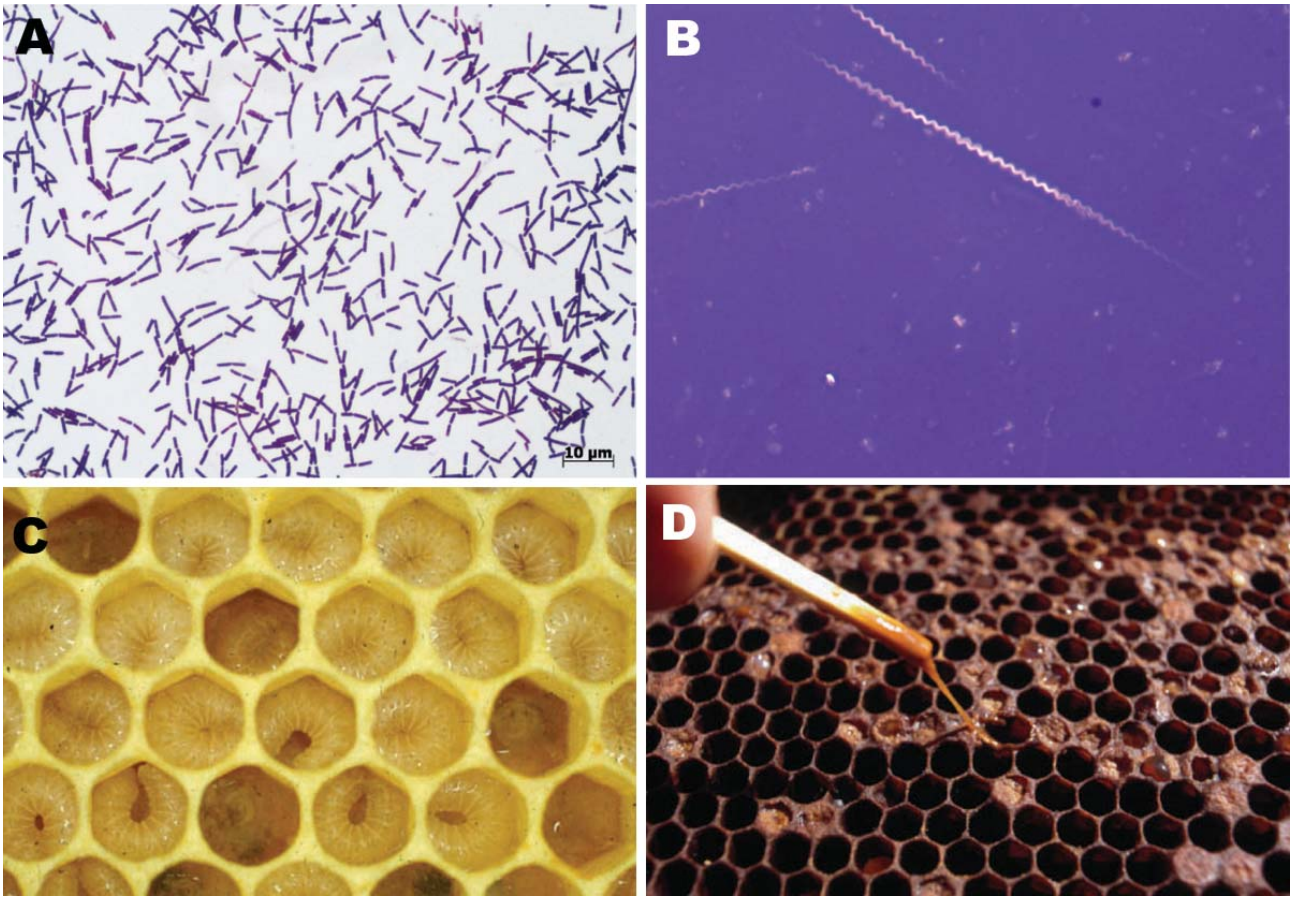

Figure. *Paenibacillus larvae* gram-positive, spore-forming, rod-shaped bacteria (A) (Gram stain, original magnification ×1,000) with the ability to form giant whips upon sporulation (B) (nigrosine stain, original magnification ×1,000). In American foulbrood (AFB), newly hatched honey bee larvae become infected through ingestion of brood honey containing *P. larvae* spores. After germination and multiplication, infected bee larvae die within a few days and are decomposed to a ropy mass, which releases millions of infective spores after desiccation. C) AFB-diseased larvae are beige or brown in color and have diminished segmentation (healthy and AFB-diseased larvae). D) Clinical diagnosis of AFB can be made by a matchstick test, demonstrating the viscous, glue-like larval remains adhering to the cell wall.

more, the novel species *P. massiliensis*, *P. sanguinis*, and *P. timonensis* were isolated from blood cultures of patients with carcinoma, interstitial nephropathy, and leukemia, respectively (*13*). Pseudobacteremia of *P. hongkongensis* and *P. macerans* has been reported (*14*,*15*).

Several aspects provide strong evidence for a genuine *P. larvae* bacteremia in the cases described here. First, the present cases were observed over a period of several years, and detection of *P. larvae* thus occurred in different charges of blood culture bottles, which argues against pseudobacteremia. Second, isolation of *P. larvae* was reported independently by 2 microbiology laboratories, making contamination highly unlikely. Third, in patient 1 isolation succeeded at different times and in samples of different compartments. Moreover, the detection of *P. larvae* in honey-prepared methadone and honey strongly suggests genuine bacteremia as a consequence of injection of contaminated material.

Biochemical and molecular identification of *P. larvae* may be difficult and time-consuming. Misinterpretation of blood culture results because of incomplete differentiation or confusion with other gram-positive spore forming-bacteria (e.g., *Bacillus* species) has to be taken into consideration. Underestimation of the frequency of true *P. larvae* bacteremia therefore cannot be excluded. Thus, infectious disease physicians, microbiologists, and pharmacists need to be aware that injection of material contaminated with *P. larvae*, such as honey-prepared methadone, may cause bacteremic infection.

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Table. Patient characteristics, clinical presentation, treatment, and laboratory and microbiologic results of 5 patients with *Paenibacillus larvae* bacteremia*

Characteristic	Patient no.				
	1	2	3	4	5
Age, y/sex	28/F	32/M	20/M	35/M	27/F
Date evaluated	2003 Jul	2003 Sep	2003 Oct	2004 Feb	2008 May
Clinical samples with identification of *P. larvae*†	Culture of ascites (2003 Jul), blood culture (2003 Aug)	Blood culture	Blood culture	Blood culture	Blood culture
CRP, mg/L	43	17	11	37	40
Leukocyte count, × 10^9/L	23.0	13.0	9.3	11.8	19.2
Medical history	IVDA, hepatitis C, Child B liver cirrhosis with refractory ascites	IVDA, hepatitis C, hepatitis B	IVDA, hepatitis C	IVDA, hepatitis C, history of hepatitis A	IVDA, hepatitis C, alcohol abuse
Clinical signs and symptoms	Decompensated liver cirrhosis, ascites, fever (39.2°C)	Persistent weakness and malaise, fever (39.2°C)	Somnolence, fever (38.2°C)	Tachypnoe, right-sided pleuritic chest pain, fever (37.8°C)	Severe anemia, spontaneous mucosal bleeding, fever (39.8°C)
Clinical conditions other than bacteremia	Bacterial peritonitis, hepatic encephalopathy after TIPS placement	Acute hepatitis B diagnosed 1 mo before bacteremia, eosinophilia	Methadone/ diazepam overdose	Pulmonary embolism, infarction pneumonia, deep vein thrombosis	Subsequently diagnosed with ITP, *Paracoccus yeei* and *Micrococcus luteus* bacteremia
Treatment (duration)	Meropenem (7 d) followed by ampicillin IV (2 d), then meropenem (7 d) followed by penicillin G (14 d)	None	None	Cefuroxim IV (7 d)	Imipenem (21 d)

*CRP, C-reactive protein (reference range <5 mg/L); IVDA, intravenous drug abuse; TIPS, transjugular intrahepatic portosystemic shunt; ITP, idiopathic thrombocytopenic purpura; IV, intravenous.
†*P. larvae* identified after culture using 16S rRNA gene sequencing.

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Rickettsia helvetica in Patient with Meningitis, Sweden, 2006

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Pathogenicity of *Rickettsia helvetica* is relatively unknown. We isolated a spotted fever group rickettsial organism from a patient with subacute meningitis. Nucleotide sequences of the 16S rRNA, *ompB*, and 17kDa genes identified the isolate as *R. helvetica*. This organism may be associated with serious infections such as central nervous system disorders.

Rickettsia helvetica, a member of the spotted fever group rickettsiae (SFGR), has been isolated from *Ixodes ricinus* ticks in many European and Asian countries. Although *I. ricinus* ticks are the main vector and natural reservoir, the organism has recently been found in *Dermacentor reticulates* ticks (*1*–*5*). Serosurveys have found antibodies reactive to *R. helvetica* in 1.9%–12.5% of the population in Lao People's Democratic Republic, France, Italy, Denmark, and Sweden (*1*,*4*,*6*–*8*). The organism has mainly been considered nonpathogenic; several patients with a serologic diagnosis have had mild, self-limited disease with associated fever, headache, and myalgia. However, a more severe clinical disease has been demonstrated (*1*,*9*).

It is well known that Q fever and the rickettsial diseases typhus and spotted fever may cause central nervous system infection and that, of the SFGR, *R. rickettsii, R. conorii*, and *R. japonica* have a documented association with meningitis (*10*,*11*). We document a case of subacute meningitis caused by *R. helvetica*. The study was reviewed and approved by the Ethics Committee, Uppsala University, Sweden.

The Case

In September 2006, a 56-year-old woman was hospitalized in Falun, Sweden, after 3 weeks of illness with gradually worsening headache and fever. She had no lymphadenopathy, rash, eschar, or history of tick bite or tick exposure. Radiographs showed a small, retrocardial pulmonary infiltrate, but the patient had few, if any, respiratory symptoms. Laboratory tests showed elevated C-reactive protein (56–128 mg/L), slightly elevated to reference-level leukocyte count (9,800–12,000 cells/μL), slightly low thrombocyte count (150,000 cells/μL), and reference values of hemoglobin and of aspartate and alanine aminotransferases. Cerebrospinal fluid (CSF) showed a slight pleocytosis (28 cells × 10^8/L, of which 18 ×10^8/L were mononuclear cells) but was otherwise within reference limits. CSF was stored at –20°C in a regular freezer and thawed only when used 1 year later. Negative results were obtained for blood and CSF cultures and for investigation for herpesviruses, tickborne encephalitis, and enteroviruses. Urine was negative for *Legionella* and pneumococcal antigens, and serum was negative for antibodies against *Borrelia burgdorferi*. Computed tomography images of the brain and sinuses were unremarkable.

Intravenous administration of cefuroxime had no effect on the fever. Because atypical pneumonia was suspected, treatment was changed after 3 days to doxycycline (100 mg 2×/day). After 2–3 days the patient's fever was gone, and she slowly recovered. The treatment was continued for 10 days. At a follow-up visit 1 year later, the patient was still well but had been asthenic for several months. No antibodies against *Mycoplasma pneumoniae* or *Coxiella burnetii* were found at the follow-up visit, and no other possible causative agent was confirmed. After giving informed consent, the patient was retrospectively included in an ongoing project that involved searching for fastidious organisms.

The patient's previously frozen CSF was divided into 2 aliquots; bacterial DNA was extracted by using a MagNa Pure Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. A genus-specific real-time PCR, as described by Stenos et al. (*12*), was used to detect SFGR. The PCR was performed in a Lightcycler 1.0 Real-Time PCR System (Roche Diagnostics GmbH) by using an LC Taqman Master Kit (Roche Diagnostics GmbH), primers, and TaqMan probe targeting the citrate synthase gene (*12*). To minimize risk for contamination, 0.25 μL LC uracil-DNA glycosylase (Roche Diagnostics GmbH) was included in each reaction. The positive control contained purified DNA of *R. helvetica* originally isolated from a domestic *I. ricinus* tick (*3*); the negative control contained sterile water. Positive samples were further analyzed by using 3 nested PCRs that amplify the 17kDa, outer membrane protein B (*ompB*), and 16S rRNA gene fragments as previously described (*3*,*13*,*14*) (Table). Amplification was conducted in a DNA thermal cycler (Hybaid, Ashford, UK) and a MJ Mini Gradient Thermal Cycler (Bio-Rad, Hercules, CA, USA), and expected fragment sizes were confirmed by gel electrophoresis in 2% agarose. Direct cycle sequencing analysis of both strands of nested PCR products was performed at the Center for Genomics and Bioinformatics, Karolinska Institutet, Stockholm, Sweden.

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DOI: 10.3201/eid1603.090184

Rickettsial DNA was amplified by real-time PCR from both CSF aliquots. Positive samples were further examined by using nested PCRs. The sequences obtained were 165 (17 kDa) and 253 bp (*ompB*) and shared 100% similarity with the corresponding gene sequences of *R. helvetica* (GenBank accession nos. EU407139, EU407140).

To isolate the pathogen, we injected CSF from the frozen aliquot in volumes of 10 μL in a 25-cm^3 flask into confluent monolayers of Vero cells and 80 μL in the other (*15*). After incubation, the cell culture was maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum and kept in a humid cell chamber in 5% CO_2, at 32°C, to allow rickettsiae to multiply. All cell lines and reagents were checked weekly for growth or bacterial contamination. Detection of growing rickettsiae was monitored by using Gimenez staining and an immunofluorescence assay of cells collected after centrifuging the medium and staining with rabbit antirickettsial hyperimmunserum and Alexa Fluor 488 goat antirabbit immunoglobulin (Ig) G (H+L) conjugate (Invitrogen, Carlsbad, CA, USA) as secondary antibody (Figure).

After 6 weeks, many intracellular bacteria were observed in the cells. Rickettsial DNA was verified by real-time PCR (*12*). The sequences obtained by nested PCR for the 17kDa and *ompB* genes in the isolate grown in Vero cells were identical to the sequences of the isolates obtained from the CSF (*3*,*13*,*14*). Amplification and partial sequencing of the 16S ribosomal RNA gene of the isolate produced fragments of 1,400 and 750 bp, respectively, which were 100% homologous to fragments of the deposited 16S ribosomal DNA sequence of *R. helvetica* from ticks (GenBank accession no. L36212).

SFGR antigen prepared from isolates grown in Vero cells of *R. helvetica* from an *I. ricinus* tick and from the patient was applied to each well of microscope slides. The antigen was dried, fixed in acetone, and incubated with serial dilutions of serum or CSF, as previously described (*7*). The positive control was serum from a patient with proven Mediterranean spotted fever and end-point IgG titers of 160 (provided by the Swedish Institute for Infectious Disease Control); the negative control was phosphate-buffered saline and serum from 3 healthy blood donors. IgG was detected by fluorescein isothiocyanate–conjugated γ-chain–specific polyclonal rabbit antihuman IgG (DakoCytomation A/S, Glostrup, Denmark). Microimmunofluorescence assay showed IgG end titers of 160 and 320 in the early-phase serum sample when the isolates of *R. helvetica* from tick and patient, respectively, were used as antigens. No antirickettsial IgG was detected in CSF when either isolate was used as antigen.

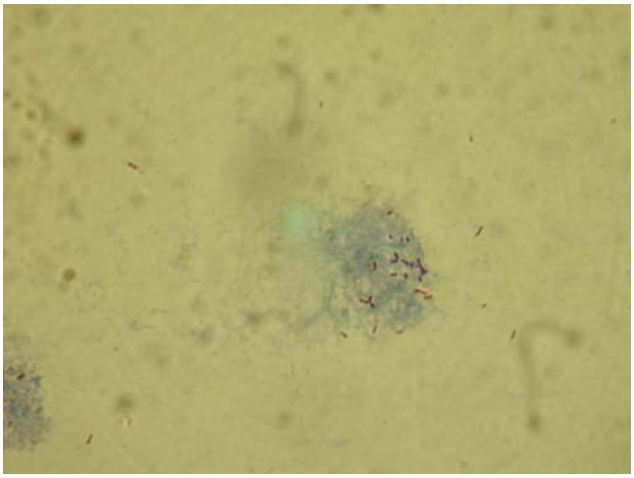

Figure. Rickettsiae in infected Vero cells. Sample from cerebrospinal fluid of patient with subacute meningitis, Sweden, 2006. Gimenez stain; original magnification ×1,000.

Conclusions

For patients with fever and headache but no rash or eschar, diagnosis is difficult and can probably not be based only on epidemiologic, clinical, and standard laboratory criteria. It therefore seems that in SFGR-endemic areas, SFGR should routinely be included in the differential diagnosis of cause of meningitis. Appropriate antimicrobial drug therapy is essential for prompt recovery and prevention of complications.

SFGR isolation is usually not available in ordinary hospital laboratories and is too time-consuming to be a di-

Table. Selected inner primers and probe used to amplify genes from cerebrospinal fluid of patient with subacute meningitis, Sweden, 2006*

Gene	Primers and probe	Nucleotide sequences, 5′ → 3′	Product size, bp
ompB	*ompB*-IF	CCAATGGCAGGACTTAGCTACT	267
	ompB-IR	AGGCTGGCTGATACACGGAGTAA	
17 kDa	RH 17-IF	GCATTACTTGGTTCTCAATTGG	214
	RH 17-IR	AACCGTAATTGCCGTTATCCGG	
16SrDNA	Ric-F	TCTAGAACGAACGCTATCGGTAT	757
	Ric-R	TTTCATCGTTTAACGGCGTGGACT	
*glt*A	SFG-CS-F	TGCCAAATGTTCACGGTACTTT	74
	SFG-CS-R	CACAATGGAAAGAAATGCACGA	
	SFG-CS-Probe	TGCAATAGCAAGAACCGTAGGCTGGATG	

**ompB*, outer membrane protein B; *gltA*, citrate synthase.

agnostic alternative in clinical settings. Although this patient's CSF had been stored in a regular freezer for 1 year, the rickettsial organisms were still viable.

PCR seems to be the most practical way to diagnose a suspected central nervous system disorder such as meningitis. The amplified nucleotide sequences were long enough to exclude other related rickettsial species. For example, the differences from other related rickettsiae were 10 and 5 nt for the *R. monacensis* 17kDa and *ompB* gene fragments, respectively, and 8 nt for *R. slovaca ompB*. Our study suggests that *R. helvetica* may cause infection of the CNS. When seeking to diagnose possible agents of meningitis, the usefulness of PCR and the relevance of the broader clinical spectrum of acute febrile illness caused by *R. helvetica* should be considered.

The study was supported in part by grants from the Center of Clinical Research Dalarna (project no. 9028).

Dr Nilsson is a physician who works in infectious disease medicine and clinical microbiology at Uppsala University Hospital. His research interests include the clinical, diagnostic, and epidemiologic features of rickettsioses.

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Rare Influenza A (H3N2) Variants with Reduced Sensitivity to Antiviral Drugs

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In 2007 and 2008 in Myanmar, we detected influenza viruses A (H3N2) that exhibited reduced sensitivity to both zanamivir and amantadine. These rare and naturally occurring viruses harbored a novel Q136K mutation in neuraminidase and S31N mutation in M2.

Adamantanes and neuraminidase inhibitors (NAIs) are the 2 classes of drugs indicated for preventing or treating influenza virus infection. In 2005, the high prevalence of influenza viruses A (H3N2) with S31N mutation in M2 limited the effectiveness of amantadine (*1*,*2*). In 2008, the emergence of subtype H1N1 with H274Y mutation in neuraminidase (NA) raised concerns about the use of oseltamivir (*3*,*4*). On the other hand, the incidence of zanamivir-resistant viruses was low (*5*). In 1998, 1 case of zanamivir-resistant influenza B virus, which was isolated from an immunocompromised child who underwent prolonged zanamivir treatment, was reported (*6*). In 2008, subtype H3N2 with D151A/V mutations in NA demonstrated reduced zanamivir sensitivity by chemiluminescent NAI assay (*5*). Recently, zanamivir-resistant subtype H1N1 isolates with a novel Q136K mutation in NA were isolated in Oceania and Southeast Asia (*7*).

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DOI: 10.3201/eid1603.091321

We report the detection of influenza viruses A (H3N2) harboring a Q136K mutation in NA and an S31N mutation in M2, which respectively confer reductions in zanamivir and amantadine susceptibility. In 2007 and 2008, we performed phenotypic and genotypic analyses in characterizing these viruses from Myanmar.

The Study

Nasopharyngeal swabs were collected from patients with influenza-like illness at Sanpya Hospital in Yangon, Myanmar, and outpatient clinics affiliated with the Department of Medical Research (Central Myanmar) in Nay Pyi Taw. Rapid test kit–positive samples were sent to Niigata University, Japan, for subsequent analyses. Virus isolation and subtyping PCR were performed as previously described (*8*). The NAI susceptibility test was performed by a fluorescence-based NA activity assay that measures the 50% inhibitory concentration (IC_{50}) by using zanamivir and oseltamivir carboxylate (*9*). All samples were assayed in duplicates in ≥2 independent experiments. A sample was considered an extreme outlier if its IC_{50} value was 10× higher than the mean values for sensitive strains with >3 interquartile range from the 25th and 75th percentiles in the box-and-whisker plot analysis (*9*). So far, all known NAI-resistant viruses are extreme outliers (*10*). Screening for S31N mutation in M2 was done by cycling probe real-time PCR (*11*). Sequencing and phylogenetic analysis of the hemagglutinin (HA) and NA genes were performed as previously described (*8*).

A total of 253 and 802 rapid test kit–positive samples were collected in Myanmar in 2007 and 2008, respectively. Of these, 64 isolates of subtype H3N2 were detected in 2007 and 211 in 2008. NAI susceptibility assay showed 1 (1.5%) isolate (A/Myanmar/M187/2007) with a zanamivir IC_{50} value of 59.72 nM, which was collected in August 2007, and 1 (0.5%) isolate (A/Myanmar/M114/2008) with a zanamivir IC_{50} of 33.37 nM, which was collected in July 2008. These isolates respectively demonstrated a 53× and 30× reduction in zanamivir susceptibility (Table) and were extreme outliers (data not shown). On the basis of cycling probe real-time PCR assay, these viruses had an S31N mutation in M2, which confers resistance to amantadine. All subtype H3N2 viruses analyzed in this study remain sensitive to oseltamivir carboxylate (Table).

Phylogenetic analysis of the HA and NA genes showed that the isolates with reduced sensitivity to zanamivir belonged to 2 distinct clusters (Figure 1). These viruses accumulated 2 and 3 amino acid (aa) substitutions in HA and 6 and 2 aa changes in NA in 2007 and 2008 (Figure 1), respectively. Epidemiologic and sequencing data did not suggest any link between the cases. Analysis of the NA

[1]These authors contributed equally to this article.

Table. Characteristics of subtype H3N2 influenza viruses with Q136K mutation in NA and S31N substitution in M2*

Strains	Passage history	NA mutation	IC_{50}s of NA inhibitors: Zanamivir, nM ± SD	Fold change	Oseltamivir, nM ± SD	Fold change	Amantadine sensitivity† (M2 mutation)
All NAI-sensitive subtype H3N2 isolates‡	MDCK2	None	1.12 ± 0.40	1	0.86 ± 0.44	1	Resistant (S31N)
A/Myanmar/M187/2007	MDCK2	Q136K	59.72 ± 3.83	53.3	0.13 ± 0.05	0.2	Resistant (S31N)
A/Myanmar/M114/2008	MDCK2	Q136K	33.37 ± 7.02	29.8	0.16 ± 0.03	0.2	Resistant (S31N)
A/Texas/131/2002§		None	1.43 ± 0.09	1.3	0.99 ± 0.09	1.2	Sensitive
A/Texas/131/2002_E119V§		E119V	5.43 ± 0.68	4.8	94.33 ± 2.06	109.7	Sensitive

*NA, neuraminidase;IC_{50}, inhibitory concentration; NAI, neuraminidase inhibitors.
†Amantadine sensitivity was based on M2 genotyping data.
‡Average IC_{50} was calculated excluding the control viruses (n = 47).
§Reference strains used as drug-sensitive and -resistant control viruses in the NAI assay.

gene showed that the isolates with reduced sensitivity to zanamivir had a glutamine (Q) to lysine (K) substitution at aa position 136. Sequence chromatograms showed a heterogeneous population of virus possessing either Q or K at position 136, with a dominant peak for the K136 mutant (Figure 2). Direct sequencing of primary samples showed a similar profile of chromatogram with a higher signal for the K136 mutant and a minor peak for the Q136 wild-type strain (Figure 2). The rest of the zanamivir-sensitive isolates in 2007 and 2008 had the Q136 genotype, and no NAI-resistant-associated mutations were detected elsewhere in the NA gene.

Conclusions

In this study, we detected a novel influenza virus A (H3N2) with Q136K mutation in NA and S31N mutation in M2, which demonstrated reduced susceptibility to both zanamivir and amantadine but remained susceptible to oseltamivir. These Q136K viruses were isolated at a low frequency (<1.5%) in Myanmar in 2007 and 2008. Phylogenetic analysis showed that these viruses were already amantadine-resistant with S31N mutation in M2. Amantadine-resistant viruses with S31N mutation have been the predominant circulating strains among subtype H3N2 viruses in Myanmar since 2005 (*8*). The Q136K substitution in NA was probably generated by spontaneous point mutation. The HA and NA gene sequences of Q136K mutants

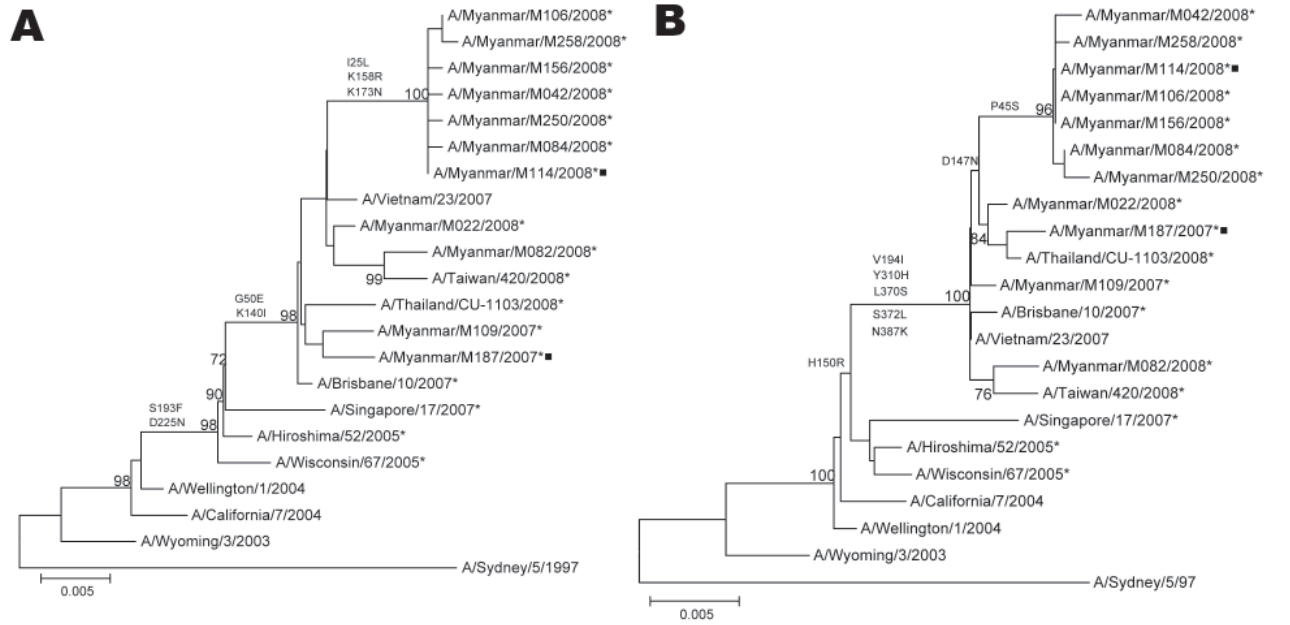

Figure 1. Phylogenetic analysis of the A) hemagglutinin (HA) and B) neuraminidase (NA) genes of influenza virus A (H3N2) isolates in Myanmar in 2007 and 2008. Trees were generated by using the neighbor-joining method. Bootstrap values >70% of 1,000 replicates and amino acid changes that characterize a branch are indicated on the left side of the node. Amantadine-resistant isolates with S31N mutation in M2 are marked with asterisks, and isolates with reduced sensitivity to zanamivir with Q136K mutation in NA are marked with squares. GenBank accession no. of the genomic sequences of isolates are GQ478849–GQ478866. Nucleotide sequences of the HA and NA genes of vaccine strains and isolates from other countries were obtained from the National Center for Biotechnology Information Influenza Virus Resource (www.ncbi.nlm.nih.gov/genomes/FLU). Scale bar indicates nucleotide substitutions per site.

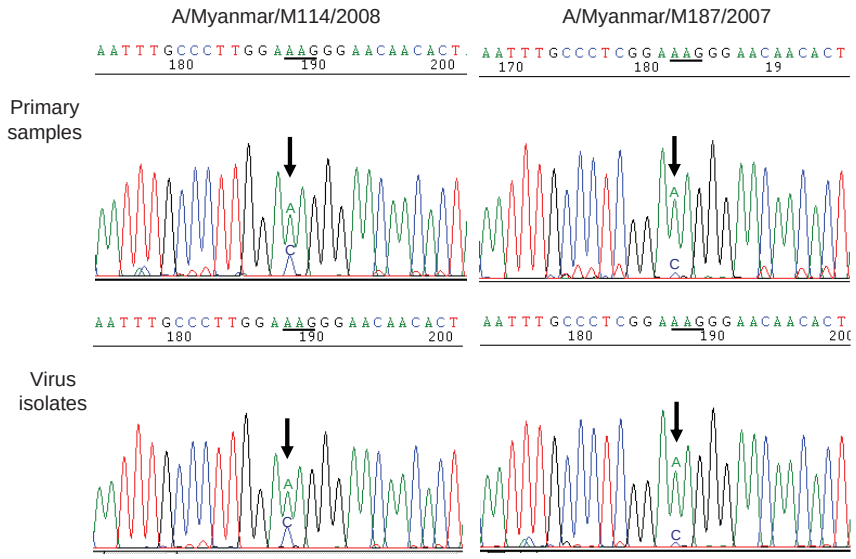

Figure 2. Detection of Q136K substitution in neuraminidase by sequencing in primary samples and virus isolates. Arrows indicate the first peak of the codon encoding amino acid position 136. Comparison of the sequence chromatogram showed a mixed population of bases in both original clinical samples and virus isolates, with a dominant peak for 136K (AAG) mutants, compared with wild-type 136Q (CAG) viruses.

were submitted to GenBank under accession nos. A/Myanmar/M187/2007: FJ229893 (HA), FJ229860 (NA) and A/Myanmar/M114/2008: GQ478854 (HA), GQ478863 (NA).

Hurt et al. recently reported the characterization of zanamivir-resistant subtype H1N1 with Q136K mutation in NA (*7*). Zanamivir IC_{50}s of these viruses ranged from 6 nM to 238 nM (*7*); which differed from the 1–60 nM range of subtype H3N2 viruses obtained in this study. This finding may be due to differences in subtype and variations in the assay. The Q136K mutation was not detected in the primary clinical samples by sequencing (*7*); however, in our study, the Q136K mutation in subtype H3N2 isolates was detected in primary samples. Comparison of the sequence chromatograms between original samples and virus isolates showed a similar profile, suggesting that the Q136K mutants were present in primary samples of subtype H3N2 isolates. The presence of Q136K variants in primary samples appears to be subtype-specific because these mutants were present in very low proportions among subtype H1N1 viruses (*12*). To determine whether mutations exist in other gene segments associated with Q136K mutations, we performed a full genome analysis of Q136K mutants and wild-type viruses. We found no additional mutations in Q136K strains, which suggest that the genetic background of these viruses can compensate for the K136 mutation. However, further study is needed to confirm whether the accumulated 5 aa changes in HA and 8 substitutions in NA would compensate for the Q136K mutation.

We searched the database for NA sequences of influenza viruses A (H3N2) with Q136K mutation that are available on GenBank. Of the 3,381 sequences obtained, 4 sequences from human influenza, which were isolated in 1995, 2003, 2004, and 2007, and 1 sequence from swine influenza, which was isolated in Japan in 1997, contained the Q136K substitution. Sequences from Q136K mutants isolated before 2007 showed no mutations in the M2 gene. The data indicate that these viruses occur naturally because some of the isolates in the database were obtained before introduction of zanamivir into clinical practice in 1999 in Australia, New Zealand, United States, and Europe (*9*,*13*). In addition, Myanmar patients who shed these Q136K viruses did not receive any NAIs. The clinical relevance of Q136K mutants is unknown. Further study is needed to evaluate the effectiveness of zanamivir in patients infected with Q136K mutants.

Continued monitoring of viruses with reduced sensitivity to NAI and adamantanes is needed, and routine surveillance should include both phenotypic and genotypic assays. The Q136K substitution in NA should be used as a molecular marker associated with reduced NAI susceptibility not only in subtype H1N1 isolates but also among subtype H3N2 isolates.

Acknowledgments

We thank the staff of the Chest Medical Unit of Sanpya Hospital, Respiratory Medicine Department of Yangon General Hospital, and Department of Medical Research for sample collection; Akemi Watanabe for excellent technical support; and Akinori Miyashita and Ryozo Kuwano for support with DNA sequencing.

This work was supported by the Special Coordination Funds for Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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etymologia

Yersinia

[yər-sin´-e-ə]

This genus of gram-negative bacteria was named after bacteriologist Alexandre-Émile-John Yersin (1863–1943). Born in Switzerland, he studied medicine in Paris and began a successful early career in the laboratory. He worked on rabies with Pierre Roux and on the tubercle bacillus under Robert Koch in Germany. He later worked at the Institut Pasteur on the toxic properties of the diphtheria bacillus and eventually signed on as a doctor on a ship headed for Saigon and Manila. In 1894, while he still worked for a French shipping company, he investigated an outbreak of plague in Hong Kong. After 7 days in a makeshift laboratory, he isolated the plague bacterium, which he called *Pasteurella pestis*.

Japanese bacteriologist Shibasaburo Kitasato had arrived in Hong Kong, a few days before Yersin and also had isolated the bacterium. Kitasato published his findings in English and Japanese. Yersin published his in French. He also established a laboratory in Nha Trang, Vietnam, where he developed an antiplague serum that reduced the death rate from 90% to ≈7%. Since 1970, the organism has been called *Yersinia pestis*.

Source: Burns W. Alexandre Yersin and his adventures in Vietnam. 2003; Medical Research Council National Institute for Medical Research. http://www.himr.mrc.ac.uk/millhillessays/2003/yersin/; http://www.whonamedit.com/doctor.cfm/2454.html; Dorland's illustrated medical dictionary, 31st ed. Philadelphia: Saunders Elsevier; 2007.

Sarcocystis Species Lethal for Domestic Pigeons

Philipp Olias, Achim D. Gruber, Andrea Kohls, Hafez M. Hafez, Alfred Otto Heydorn, Heinz Mehlhorn, and Michael Lierz[1]

A large number of *Sarcocystis* spp. infect birds as intermediate hosts, but pigeons are rarely affected. We identified a novel *Sarcocystis* sp. that causes lethal neurologic disease in domestic pigeons in Germany. Experimental infections indicated transmission by northern goshawks, and sequence analyses indicated transnational distribution. Worldwide spread is possible.

A large number of *Sarcocystis* spp. (Protozoa: Apicomplexa) may infect birds as intermediate hosts, but wild Columbiformes, which include pigeons, are rarely affected (*1*–*3*). Among the few species affecting domestic poultry are *S. horvathi* and *S. wenzeli*, which affect chickens, and *S. rileyi*, for which ducks are intermediate hosts (*4*,*5*). *S. falcatula* has been known to cause clinical disease in pigeons only after experimental infection; whether this species is pathogenic under natural conditions is not known (*6*).

We recently reported an emerging neurologic disease with lethal outcome for domestic pigeons (*Columba livia* f. *domestica*) in Berlin, Germany, caused by a novel *Sarcocystis* sp. (*3*). When compared with *S. falcatula* and other bird-infecting *Sarcocystis* spp. such as *S. lindsayi*, the novel species differed in its ultrastructural and genetic features (*3*,*6*,*7*). Clinical signs in naturally infected pigeons, which were similar to those caused by *Paramyxovirus*-1 or *Salmonella typhimurium* var. cop. infection, were depression, polyuria, torticollis, opisthotonus, paralysis, trembling, and death. Pigeons had numerous parasitic cysts in their muscles. We hypothesized that pigeons serve as intermediate hosts in a 2-host life cycle characteristic for *Sarcocystis* spp., in which pigeons are infected by ingestion of sporocysts shed in feces from an unidentified definitive host (*8*). We further characterized the parasite genetically, identified its definitive host and life cycle, and determined its causative role in this novel disease of pigeons.

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DOI: 10.3201/eid1603.090860

The Study

In 2008, DNA was extracted from the pectoral muscles of a pigeon that had been naturally infected during a recent outbreak in Germany. DNA sequences encoding the 18S rRNA and D2-region of the 28S rRNA of the *Sarcocystis* sp. were PCR amplified and sequenced, after which multiple sequence alignments and construction of phylogenetic relationships were conducted (*3*,*9*–*11*). The 18S rRNA and D2-region sequences were deposited in the GenBank database (accession no. GQ245670). Comparison of the 18S rRNA with published sequences of *Sarcocystis* spp. identified 1 matching sequence of 783 bp isolated from a Cooper's hawk (*Accipiter cooperii*) (GenBank accession no. EU810398). Sequence analysis of a combination of the 18S rRNA and D2 region showed close homologies to other bird-infecting *Sarcocystis* spp. (Figure 1) and only 4 nt differences from a *Sarcocystis* sp. found in a white-fronted goose (*Anser albifrons*) (*12*).

To identify the definitive host, we conducted an experimental infection study using predators that had possible contact with the naturally infected pigeons: 2 dogs (*Canis familaris*, beagles), 2 ferrets (*Mustela putorius furo*), 2 rats (*Rattus norvegicus* f. *domestica*), 2 mice (*Mus musculus domesticus*), 2 northern goshawks (*Accipiter gentilis*), and 2 Gyr-Saker hybrid falcons (*Falco rusticolus* × *Falco cherrug*). Fecal samples from all animals were negative for parasites before infection. Each animal was fed 1 regular-sized meal of pectoral muscle of 2 racing pigeons naturally infected with cysts from the 2008 outbreak in Germany (*3*). Starting on day 6 after infection, only the goshawks shed sporocysts (7.9 × 11.9 μm) in their feces. Microscopically, many oocysts (each containing 2 sporocysts) were detected in the mucosa of the small intestine, which is characteristic for *Sarcocystis* spp. Identical D2-region sequences were detected in sporocysts from goshawk feces and in *Sarcocystis*-infested muscles from naturally infected pigeons. All other animals failed to shed sporocysts. No clinical signs developed in the goshawks or the other animal species.

To experimentally reproduce the disease, we infected domestic pigeons with an oral dose of purified sporocysts from 1 goshawk. Pectoral muscle biopsy samples taken before experimental infections were free of parasites. Fecal examination confirmed absence of *Salmonella* spp. and endoparasites. We separated 16 pigeons into 8 groups of 2 birds each and gave pigeons in groups 1–7 infectious doses (IDs) of 3×10^6, 3×10^5, 10^5, 8×10^4, 10^4, 10^3, or 10^2. Pigeons in group 8 served as controls. Animals with neurologic signs were euthanized, and surviving pigeons were euthanized at 59 and 120 days after infection, respectively.

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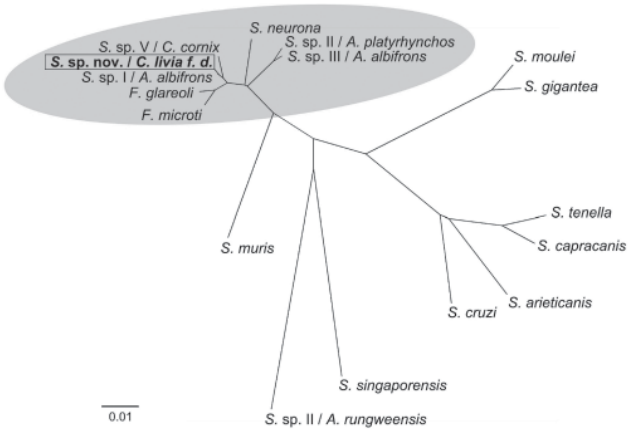

Figure 1. Phylogenetic comparison of novel *Sarcocystis* sp. with related *Sarcocystis* spp. Tree constructed by neighbor-joining using Kimura 2-parameter method based on the partial 18S rRNA gene comprising 1,391 bp and the D2 region of 28S rRNA gene comprising 325 bp of the novel *Sarcocytis* sp. (GenBank accession no. GQ245670/FJ232949) and the following available *Sarcocystis* sequences: *Frenkelia microti* (*S. buteonis*) (AF009244/AF044252); *Frenkelia glareoli* (*S. glareoli*) (AF009245/AF044251); *S*. sp. (cyst type I) ex *Anser albifrons* (EU502869/EF079886); *S*. sp. (cyst type V) ex *Corvus cornix* (EU553478/EF079884); *S. neurona* (U07812/AF092927); *S*. sp. (cyst type II) ex *Anas platyrhynchos* (EU553477/EF079887); *S*. sp. (cyst type III) ex *A. albifrons* (EU502868/EF079885); *S. moulei* (L76473/AF012884); *S. gigantea* (L24384/U85706); *S. tenella* (L24383/AF076899); *S. capracanis* (L76472/AF012885); *S. arieticanis* (L24382/AF076904); *S. cruzi* (AF017120/AF076903); *S. singaporensis* (AF434054/AF237617); *S*. sp. II ex *Atheris rungweensis* (AF513490/AF513493); *S. muris* (M64244/AF012883). Highlighted area indicates branch of bird-infecting *Sarcocystis* spp. Scale bar indicates genetic distance.

Pigeons in groups 1–4 (IDs 3×10^6 to 8×10^4) died within 12 days after infection. After 8 weeks of infection, severe and moderate neurologic signs developed in pigeons of groups 5 (ID 10^4) and group 6 (ID 10^3), respectively. After 9 weeks of infection, pigeons in group 7 (ID 10^2) had mild to moderate neurologic signs. Control pigeons of group 8 remained free of clinical signs throughout the study.

Histologic examination of livers from pigeons in groups 1–4 showed multifocal severe necroses with numerous parasitic stages (Figure 2, panel A). Pigeons in groups 5–7 had marked encephalitis, myositis, and *Sarcocystis* cysts in skeletal muscles (pectoral, gastrocnemius, and neck) but not in the brain. Control pigeons had no microscopic lesions in any organs. Neither *Salmonella* spp. nor a hemagglutinating agent was cultured from any pigeon.

Electron microscopic examination of livers was performed as previously described (*3*). Parasitic stages, identified as developmental stages of schizonts, were seen in livers of pigeons of groups 1–4 (Figure 2, panel B). Simultaneous development of merozoites above a giant nucleus of the schizont, the typical endopolygeny for a *Sarcocystis* parasite, was noted. Identical D2-region DNA sequences were detected in the livers and skeletal muscles from all experimentally infected pigeons and from the *Sarcocystis*-infested muscles from naturally infected pigeons.

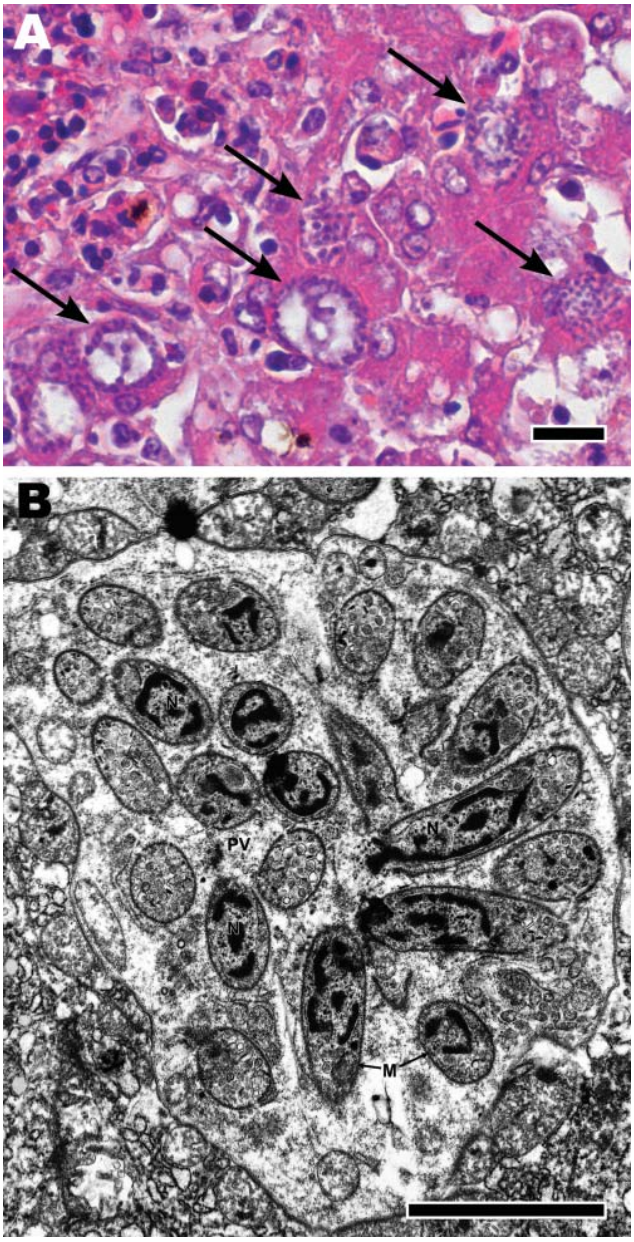

Figure 2. A) Microscopic appearance of liver with tissue necrosis, lymphohistiocytic inflammation, and *Sarcocystis* schizonts (arrows) in a pigeon 8 days after infection with 10^5 *Sarcocystis* sporocysts. Hematoxylin and eosin stain; scale bar = 20 μm. B) Transmission electron micrograph of a hepatocyte from liver in panel A, containing a schizont, forming cross-sectioned and longitudinally sectioned merozoites. N, nucleus; PV, parasitophorous vacuole; M, merozoite. Scale bar = 20 μm.

Conclusions

This study identifies the northern goshawk as the probable definitive host of a recently described novel *Sarcocystis* sp. in domestic pigeons in Germany, indicating a typical prey–predator transmission cycle (*3*). The clinical signs and organ lesions of experimentally infected animals mirror those of naturally infected racing pigeons.

Previous results suggested that this parasite represents a new *Sarcocystis* sp. and is genetically distinct from *S. falcatula* (*3*). Our further sequence analyses indicated that the novel *Sarcocystis* sp. is closely related or even identical to a *Sarcocystis* sp. previously detected in a Cooper's hawk in the state of Georgia, USA (*13*). Cooper's hawks are widespread in North America and in some areas hunt mainly pigeons (*14*). Further phylogenetic analyses showed that this *Sarcocystis* sp. is closely related but distinct from other bird-infecting *Sarcocystis* spp. (Figure 1).

Goshawks are widely distributed in the Northern Hemisphere, where the domestic pigeon is also common. Throughout Europe, pigeons are the principal prey for goshawks (*15*). Thus, we speculate that this *Sarcocystis* sp. may be present in other countries or could easily be introduced and become endemic elsewhere. It remains to be shown whether other avian species, in addition to pigeons, may serve as intermediate hosts. This assumption is supported by a close sequence homology between this *Sarcocystis* sp. and a *Sarcocystis* sp. previously found in striated muscles of a white-fronted goose (Figure 1).

Among the experimentally infected pigeons, different diseases were caused by different infectious doses. Pigeons infected with high doses died 7–12 days after infection and had massive parasite-induced liver necroses; those infected with lower doses had central nervous signs, which did not develop until 8 weeks after infection. The late occurrence of brain lesions and the absence of parasitic stages from the brain suggest an indirect, currently unknown, mechanism of encephalitis that awaits further clarification.

In conclusion, the emerging *Sarcocystis* sp. cycles between northern goshawks and domestic pigeons and is highly pathogenic for the pigeons after they ingest low doses of sporocysts. Pigeon sport and falconry should therefore be considered as risk factors for further disease transmission.

Dr Olias is a PhD student in the Department of Veterinary Pathology at the Freie Universität Berlin, Germany. Avian diseases are his primary research interest.

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Candidatus Bartonella mayotimonensis and Endocarditis

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We describe a new *Bartonella* species for which we propose the name *Candidatus* Bartonella mayotimonensis. It was isolated from native aortic valve tissue of a person with infective endocarditis. The new species was identified by using PCR amplification and sequencing of 5 genes (16S rRNA gene, *ftsZ*, *rpoB*, *gltA*, and internal transcribed spacer region).

Bartonella species are small, fastidious, gram-negative, intracellular bacteria that cause culture-negative infective endocarditis. Six species have been documented to cause endocarditis in humans: *B. quintana* (*1*), *B. henselae* (*2*), *B. elizabethae* (*3*), *B. vinsonii* subsp. *berkhoffii* (*4*), *B. koehlerae* (*5*), and *B. alsatica* (*6*). We report a case of culture-negative endocarditis caused by a new *Bartonella* species, for which we propose the name *Candidatus* Bartonella mayotimonensis.

The Patient

A 59-year-old man was initially hospitalized at Sartori Memorial Hospital (Cedar Falls, IA, USA) from April 14 through 19, 2008, for progressive shortness of breath, weight loss, fatigue, and altered mental status. He was then transferred to the Mayo Clinic (Rochester, MN, USA). Physical examination identified a new diastolic heart murmur. He was afebrile and did not have peripheral stigmata of endocarditis. Two sets of blood cultures obtained before antimicrobial drug therapy showed negative results for all bacteria tested after 5 days of incubation. A transesophageal echocardiogram showed a bicuspid aortic valve, mobile components on the left cusp of the aortic valve suggesting vegetations, and a 5.3-cm ascending aortic aneurysm. Empiric antimicrobial drug therapy, including vancomycin and ceftriaxone, was initiated. Subsequently, acute renal dysfunction, possibly secondary to vancomycin exposure, developed in the patient.

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DOI: 10.3201/eid1603.081673

The patient lived alone on a farm in Iowa, USA, and had not had recent exposure to animals. However, he had observed murine fecal droppings in his house and mice on the farm. He had had a house cat for 18 years until its death a few years before his hospitalization and had intermittent contact with cats when he visited his daughter.

Serum immunoglobulin G titers were positive for *B. henselae* and *B. quintana* (≥1,024). Oral doxycycline and rifampin were prescribed for treatment of presumed *Bartonella* endocarditis. Gentamicin was not administered because of development of the acute renal dysfunction. Two weeks later, he underwent aortic valve and aortic root replacement. Results of gram staining, acid-fast staining, fungal staining, anaerobic bacterial culture, aerobic bacterial culture, mycobacterial culture, and fungal culture on resected aortic valve tissue were negative for *Bartonella* species.

PCR performed at the Mayo Clinic on resected aortic valve tissue detected part of the citrate synthase gene (*gltA*) of *Bartonella* species. However, the melting temperature was not characteristic of *B. quintana* or *B. henselae* (*7*). Oral doxycycline and rifampin were continued for 12 weeks after aortic valve resection. The patient was well and had no signs of relapsing infection at a follow-up visit 11 months after valve surgery.

Aortic valve tissue and serum were tested at the Unité des Rickettsies, Marseille, France. *B. quintana* Oklahoma, *B. henselae* Houston (ATCC 49882), *B. vinsonii* subsp. *berkhoffii* (URBVAIE25), *B. vinsonii* subsp. *arupensis* (ATCC 700727), and *B. alsatica* (CIP 105477 T) strains were used for immunofluorescent assays and Western blotting (*6*). Valve tissue was injected into human endothelial cells in a shell vial assay and onto Columbia 5% sheep blood agar plates and incubated at 37°C in an atmosphere of 5% CO_2 as described (*6*).

A *Bartonella* species was detected in a shell vial by immunofluorescence after 15 days of culture; identification was confirmed by PCR. DNA was extracted from valve specimen and injected cells by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany). DNA was used as a template in a genus *Bartonella* Lightcycler assay with primers and a Taqman probe specific for the internal transcribed spacer (ITS) gene (*6*) and in standard PCR assays specific for the 16S rRNA, ITS, *rpoB*, *gltA*, and *ftsZ* genes (*8*). Sequences from both DNA strands were determined twice for all PCR products. These products were resolved in an ABI 3100 automated sequencer (PerkinElmer, Waltham, MA, USA). Sterile water was used as a negative control in each assay. Percentages of similarity among sequences were determined by using MEGA 2.1 software (*9*). Phylogenetic relationships among *Bartonella* strains were inferred from concatenated sequences by using MEGA 2.1 software (*9*).

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Surgically resected aortic valve tissues were fixed in formalin, embedded in paraffin, and sectioned to a thickness of 5 μm. Sections were stained with periodic acid–Schiff, Giemsa, Gram, Grocott-Gomori methenamine silver, and Warthin-Starry stains. Immunohistochemical analysis was performed by using a procedure described elsewhere (*10*) and polyclonal antibody against *B. vinsonii* at a dilution of 1:1,000.

Serum samples showed immunoglobulin G endpoint titers of 50 against all *Bartonella* species tested by immunofluorescent assay. Western blot results were positive and characteristic of *Bartonella* infection (Figure 1, panel A). Results of PCR (*Bartonella* genus Lightcycler assay and standard PCR for cardiac valve) and cell culture were positive, and amplification products of the expected size were obtained. Among known validated species, sequences obtained shared 99.1% (1,438/1,445 bp) homology with *B. tribocorum*, *B. henselae*, and *B. vinsonii* for the 16S rRNA gene, 89.5% homology with *B. grahamii* for the ITS gene, 93.4% homology with *B. vinsonii* subsp. *berkhoffii* for *rpoB*, 91.7% homology with *B. vinsonii* subsp. *berkhoffii* for *ftsZ*, and 92.5% homology with *B. vinsonii* strain Baker for *gltA*. The phylogenetic position of *Candidatus* B. mayotimonensis among members of the genus *Bartonella* based on comparisons of concatenated sequences of the 5 genes is shown in Figure 2. Sequences of *gltA,* 16S rDNA, *ftsZ,* ITS, and *rpoB* were deposited in GenBank under accession nos. FJ376732–FJ376736.

Histologic analysis of resected aortic valve showed infective endocarditis with vegetation containing microorganisms that stained with Warthin-Starry and Giemsa. Warthin-Starry staining showed darkly stained bacilli consistent with *Bartonella* species (Figure 1, panel B). Results of staining with periodic acid–Schiff, Gram, and Grocott-

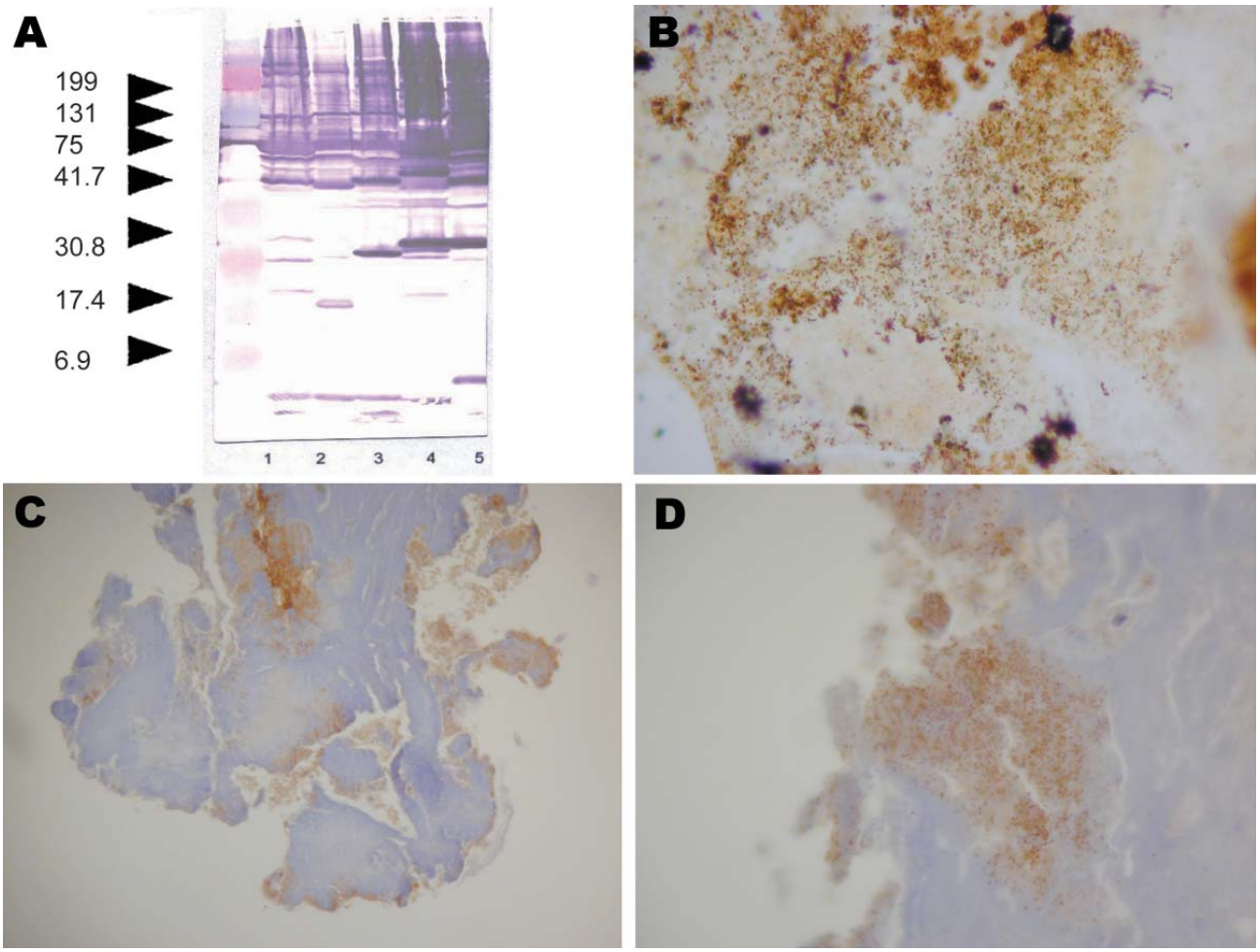

Figure 1. A) Western blot of serum sample from patient infected with *Candidatus* Bartonella mayotimonensis. Left lane, Molecular mass standard; lane 1, *Bartonella quintana*; lane 2, *B. henselae*; lane 3, *B. elizabethae*; lane 4, *B. vinsonii* subsp. *berkhoffii*; lane 5, *B. alsatica*. Values on the left are in kilobases. B) Numerous darkly stained bacilli consistent with *Bartonella* species organized in clusters in the valvular vegetation (Warthin-Starry stain; original magnification ×400). C and D) Bacteria detected by immunohistochemical analysis of an extracellular location inside the valvular vegetation (polyclonal antibody against *B. vinsonii* subsp. *berkhoffii*, Warthin-Starry stain and hematoxylin counterstain; original magnification ×100 in C and ×400 in D).

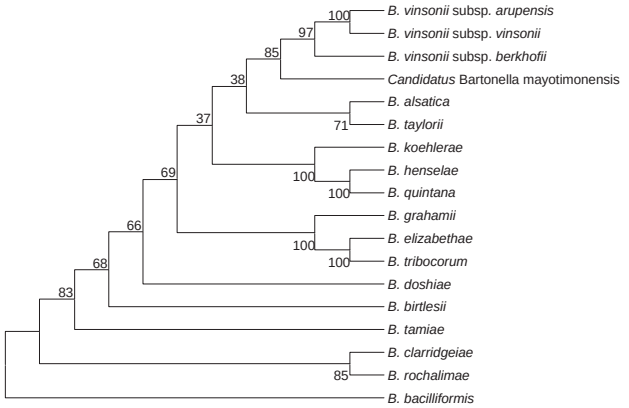

Figure 2. Phylogenetic tree showing the position of *Candidatus* Bartonella mayotimonensis among members of the genus *Bartonella* based on comparisons of concatenated sequences of the 16S rRNA gene, the citrate synthase gene *gltA*, the RNA polymerase β-subunit gene *rpoB*, the cell division gene *ftsZ*, and the 16S–23S rRNA internal transcribed spacer region sequences. The tree was constructed by using the neighbor-joining method and a maximum-likelihood–based distance algorithm. Numbers on branches indicate bootstrap values derived from 500 replications.

Gomori methenamine silver were negative. Immunohistochemical analysis detected bacteria in valvular vegetations in a location superimposable with that detected by Warthin-Starry staining (Figure 1, panels C, D).

Conclusions

We isolated a new *Bartonella* species and propose that it be named *Candidatus* Bartonella mayotimonensis to recognize the contributing institutions (Mayo Clinic and Hôpital de la Timone, Marseille, France). This is the seventh *Bartonella* species documented to cause infective endocarditis in humans.

The reservoir of *Candidatus* B. mayotimonensis has yet to be determined. Different *Bartonella* species have been isolated from a variety of mammals, and each species is highly adapted to its reservoir host (*11*,*12*). The domestic cat is the primary mammalian reservoir for *B. henselae* (*13*). Other *Bartonella* species have been found in mammalian hosts, including rats (*B. elizabethae*), dogs and coyotes (*B. vinsonii* subsp. *berkhoffii*), cats (*B. koehlerae*), humans (*B. bacilliformis* and *B. quintana*), moles (*B. talpae*), voles (*B. vinsonii* subsp. *vinsonii*), cows (*B. bovis* [*weissii*]), deer (*B. schoenbuchensis*), and rabbits (*B. alsatica*) (*3*–*6*,*12*,*14*,*15*). Our patient had direct exposure to mice on his farm and also had intermittent contact with cats while visiting his daughter. Additional investigations are needed to determine the reservoir(s) and vector(s) for this novel bacterium.

The immunofluorescent assay, the current serologic method for diagnosis of *Bartonella* infection, does not distinguish among *Bartonella* species. Only Western blot analysis and cross-adsorption enable serologic identification of species. PCR and culture are critical when a *Bartonella* species is identified for the first time as a human pathogen. Newly encountered *Bartonella* strains should be considered a new species if a 327-bp *glt*A fragment shares <96.0% sequence similarity with those of validated species, and if an 825-bp *rpo*B fragment shares <95.4% sequence similarity with those of validated species as reported in the current case (*8*).

This case reinforces the hypothesis that any *Bartonella* species can cause human infection, including culture-negative endocarditis. *Candidatus* B. mayotimonensis should be added to the list of human pathogens that can cause culture-negative endocarditis.

Dr Lin was an internal medicine resident at the Mayo Clinic in Rochester, Minnesota, when the patient's condition was investigated. She is currently an endocrinology fellow at Massachusetts General Hospital in Boston, Massachusetts. Her research interest is neuroendocrinology.

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Surveillance Lessons from First-wave Pandemic (H1N1) 2009, Northern California, USA

Roger Baxter

After the appearance of pandemic (H1N1) 2009 in April 2009, influenza activity was monitored within the Kaiser Permanente Northern California division by using laboratory, pharmacy, telephone calls, and utilization (services patients received) data. A combination of testing and utilization data showed a pattern of disease activity, but this pattern may have been affected by public perception of the epidemic.

In April 2009, the novel swine-origin H1N1 influenza virus, now referred to as pandemic (H1N1) 2009 virus, was identified in the United States in California and in Mexico. During April, increasing numbers of cases were identified in Mexico, and sporadic cases were seen in the United States, mostly in returning travelers (*1*). Media coverage was high, and the public and medical communities were alert to the presence of the novel virus (*2*). Although the World Health Organization raised its influenza alert level to phase 6 (calling this a true pandemic) on June 11, by this time media attention in the United States had waned, and concern was for reemergence in the fall (*3*). However, virus activity did not diminish in northern California; rather, pandemic (H1N1) 2009 influenza remained active at high levels.

Kaiser Permanente (KP) is a medical care organization with 3.2 million members in its Northern California division (KPNC). Members receive essentially all medical care from KP providers and in KP facilities. An electronic medical record system records diagnoses from outpatient and emergency department visits and hospitalizations, as well as medications, immunizations, and ancillary services received by patients. A central laboratory in Berkeley performs all microbiologic and virologic testing. In addition, all telephone callers to the system are routed to central call centers, where information is gathered on whether the caller is asking influenza-related questions. This report details the recent experience of pandemic (H1N1) 2009 in KPNC and documents KP surveillance efforts.

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DOI: 10.3201/eid1603.091285

The Study

Influenza testing was performed by using a real-time PCR for influenza A and B and respiratory syncytial virus on nasopharyngeal swabs. Similar methods have been shown to be superior to other tests and sensitive and specific for detecting pandemic (H1N1) 2009 influenza (*4*). A weekly report went to primary care providers, advising on current viral activity, and gave guidelines for testing and treating. All specimens positive for influenza A were transported to the California State Department of Public Health laboratory for H1N1 confirmation testing early in the pandemic, but testing was later restricted to specimens from hospitalized patients only. Results of testing were provided weekly with counts from the previous week, Sunday through Saturday. Hospitalization rates were counted weekly by using text strings from admission diagnoses for pneumonia or influenza. If KPNC members had questions regarding influenza, when they called for advice or appointments they were triaged to the "flu queue," where they could receive prerecorded messages or one-on-one advice with a nurse or physician. We plotted the percentage of all calls per week that were counted as influenza related. Weekly counts of medical office visits for influenza-like illness (ILI)—fever, influenza, or upper respiratory infection—were also plotted. The study was reviewed and approved by the Kaiser Permanente and Institutional Review Boards.

The Figure, panel A, shows that the total number of respiratory tests rose drastically during late April, when media coverage was high. This increase was accompanied by an increase in the total number of respiratory specimens positive for influenza A. During this initial phase, utilization of resources was high, but there appeared to be little pandemic (H1N1) 2009 in the community because the percentage of positive specimens ranged from 5% to 7%. The Figure, panel B, shows outpatient visits for ILI per 1,000 members and percentages of total hospitalizations for pneumonia or influenza during 2009, along with the percentage of respiratory specimens positive for influenza A. The first increase correlates with 2008–09 seasonal influenza, which peaked in February. Then in late April, at the same time as the increase in volume of influenza testing, there was an increase in outpatient visits for ILI and hospitalizations for pneumonia or influenza. In the Figure, panel C, the percentage of influenza-related telephone calls is plotted alongside the percentage of respiratory specimens that were positive for influenza A. Similarly to trends in medical appointments and hospitalizations, calls showed a marked increase during the pandemic scare period, then decreased and rose again more gradually with the first wave of the pandemic, along with the percentage of specimens positive for influenza A.

Media coverage rapidly subsided, and reports from the Centers for Disease Control and Prevention showed that the number of cases of pandemic (H1N1) 2009 was diminish-

ing in Mexico and the United States (*1*). Testing and treating diminished and utilization of healthcare services returned to normal. However, pandemic (H1N1) 2009 continued to circulate widely, even after schools closed for summer vacation. By mid-May, the percentage of specimens positive for influenza A was 10% and then rapidly increased to 49% only 4 weeks later. State subtyping of hospitalized patients (inside and outside the KP) who were positive for influenza A showed that >95% of specimens tested from those patients were either not subtypeable or were positive for pandemic (H1N1) 2009 influenza virus (J. Louie, California Department of Public Health, pers. comm.). Hospitalizations for pneumonia and influenza, outpatient visits for ILI, and influenza-related phone calls all rose in concert with the percentage of positive specimens.

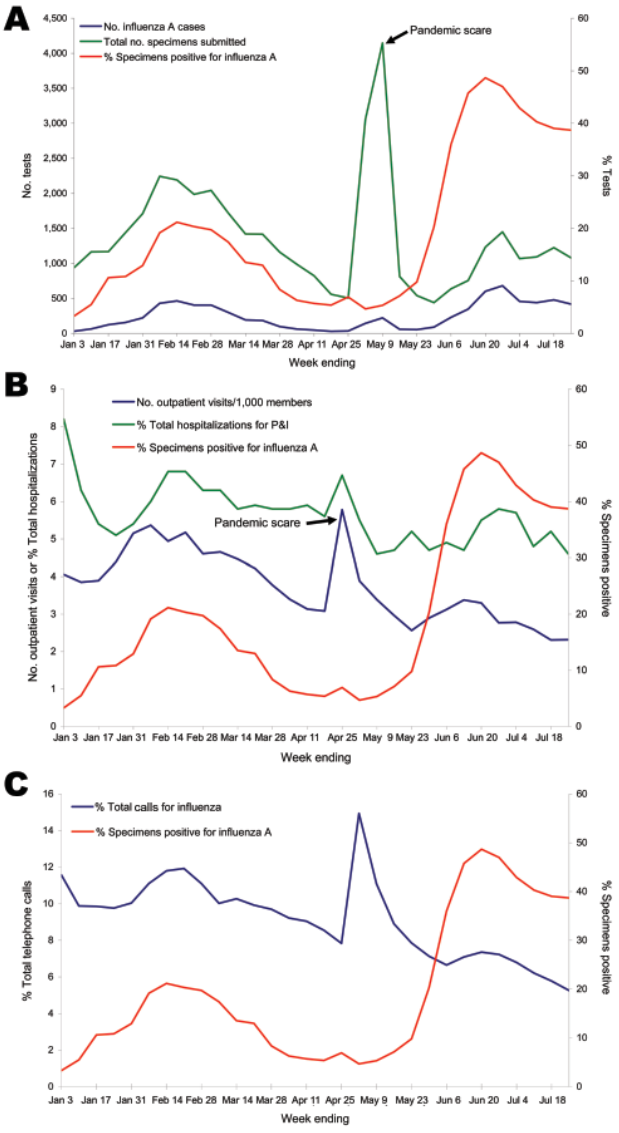

Figure. A) Influenza A testing in Kaiser Permanente Northern California division (KPNC), 2009. Shown are total numbers of specimens sent, number of specimens positive for influenza A, and percentage of specimens positive for influenza A. B) Outpatient visits for influenza-like illness (fever, influenza, or upper respiratory infection) per 1,000 members, percentage of all hospitalizations with a diagnosis of pneumonia or influenza (P&I), and percentage of specimens positive for influenza A, KPNC, 2009. C) Influenza-related telephone calls to KPNC, 2009, and percentage of specimens positive for influenza A.

Conclusions

During the recent outbreak of pandemic (H1N1) 2009 influenza in California, KPNC providers had access to quality, real-time information on the ongoing outbreak. This accessibility proved useful for guiding testing and treating algorithms and provided information during a time of great uncertainty and public fear.

Although the data were useful, it appears that during a time of intense media attention healthcare utilization may be susceptible to public perception and media coverage. During the pandemic scare period, although it appeared that influenza was circulating widely, test results and utilization data indicate that most activity was not related to either pandemic or seasonal influenza but that it may have been generated by demand created by false perceptions. It is interesting that even hospitalizations increased during this time because we generally perceive increased hospitalizations to be a marker of virulence and true activity. During the later phase of the pandemic, hospital and outpatient utilization rose in concert with the percentage of positive test results, reflecting virus activity. During this time, media coverage was relatively low, and this was reflected by lower numbers of telephone calls to the system.

The percentage of positive specimens appeared to be the best indicator of influenza activity because it was sensitive to rapid changes, but was a more specific indicator than specimens sent, number positive, outpatient or inpatient utilization, or telephone call-ins. The total number of patients tested is also informative because it can help define the relationship of testing to public perceptions. However, extremely high numbers can obscure a higher percent positive if persons seek medical care more from panic than for actual symptoms. The first-wave pandemic peak of positive samples was high compared with those from the seasonal influenza outbreak in February (49% vs. 22%); total numbers were lower. This difference may reflect patterns of testing by providers and reasons for patients to go to medical centers, but the high percentage of positive samples may reflect large numbers of cases in the community and the wide distribution of pandemic (H1N1) 2009 influenza.

The weekly report influenced provider testing with guidelines that changed as the season progressed. When the percentage positive was high at all facilities and the laboratory was overwhelmed with requests, providers were advised to decrease testing unless needed for a clinical

workup or for any hospitalization. This request may have produced artifacts in the testing in that total numbers and the percentage positive may have varied based on the sensitivity and specificity of provider testing.

Surveillance for influenza, both seasonal and pandemic, by using electronic data is informative for medical organizations with a systematic approach to testing for influenza virus. Monitoring of medical utilization may be helpful in a pandemic, but fluctuations are susceptible to public impression and media coverage. An integrated approach to influenza surveillance, combining laboratory testing and utilization, would be optimal.

Acknowledgments

I thank Lesley Levine for help with call center data, John Chapman for hospitalization data, Janice Louie and Hugo Guevara for subtyping data, Janelle Lee for outpatient data, and Bruce Fireman for encouragement.

Dr Baxter is an internist and practicing infectious diseases specialist with The Kaiser Permanente Medical Group in Oakland, California, and is co-director of the Kaiser Permanente Vaccine Study Center. His research interests include influenza vaccine effectiveness and vaccine safety.

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Experimental Infection of Squirrel Monkeys with Nipah Virus

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We infected squirrel monkeys (*Saimiri sciureus*) with Nipah virus to determine the monkeys' suitability for use as primate models in preclinical testing of preventive and therapeutic treatments. Infection of squirrel monkeys through intravenous injection was followed by high death rates associated with acute neurologic and respiratory illness and viral RNA and antigen production.

Nipah virus (NiV) is a highly pathogenic zoonotic paramyxovirus that was first identified in Malaysia and Singapore in 1999 (*1*). Since the initial outbreak, NiV has been associated with human illness in Bangladesh and India (*2*) and was classified, together with the closely related Hendra virus, in the genus *Henipavirus*. Reported human death rates varied from 40%–92% (*3*), and some outbreaks were associated with human-to-human transmission (*4*). Most human infections led to encephalitis with vasculitis-induced thrombosis in the brain and atypical pneumonia in certain patients (*5*,*6*). Because of the lack of efficient treatment or a vaccine for Nipah virus and the high pathogenicity of the virus in humans, the manipulation of NiV requires BioSafety Level 4 (BSL-4) conditions.

Several species of fruit bats of the genus *Pteropus* are considered natural reservoirs of henipaviruses, although the disease does not develop in them (*7*). Pigs were responsible for amplifying the NiV infection in Malaysia, but their death rate was only 10%–15%. Laboratory infection of piglets caused development of neurologic signs in some animals, and NiV was detected in different tissues (*8*). Hamsters in laboratory studies are highly susceptible to NiV, and infection develops in multiple organs, including the brain (*9*). Cats infected with NiV in the laboratory reproduce the disease observed in naturally infected cats, including a severe respiratory and systemic disease, 6–13 days after infection (*10*). However, to our knowledge, a primate model necessary for preclinical testing of preventive and therapeutic approaches has not been described. We therefore assessed the squirrel monkey (*Saimiri sciureus*) as an experimental model of NiV infection.

The Study

We selected these New World monkeys because of their availability, reliability as a primate model with which to study infectious diseases (*11*), and suitability as experimental animals in BSL-4 conditions. Thirteen 4-year-old male monkeys (0.8–1.0 kg) were imported from a breeding colony in French Guiana and housed in the BSL-4 animal care facility in Lyon. Experimental methods were approved by the Région Rhône Alpes ethics committee.

Twelve monkeys were infected with NiV isolate UMMC1 (*1*), GenBank accession no. AY029767, either intravenously or intranasally; for both modes of infection either 10^3 or 10^7 PFU was used. Animals were observed daily for 2 months for signs of disease onset; tissues were taken during the infection and at necropsy or at the end of experiment (Table 1). Blood samples were collected at different time points, serum samples were used for antibody analysis, and peripheral blood cells (PBMC) were used for RNA isolation. Different organ samples were taken and frozen at –80°C for RNA isolation or fixed in 4% formalin for histopathologic studies.

RNA was extracted from different organs and analyzed by 1-step RT-PCR by using high fidelity PCR enzyme blend (Roche Applied Science, Mannheim, Germany) for NiV nucleoprotein expression as described (*12*). Detection of NiV-specific antibodies in the serum was performed simultaneously for all samples by ELISA and virus neutralization assays as described (*13*). Immunohistochemical analysis was conducted on formalin-fixed, paraffin-embedded tissues as described (*6*).

Onset of clinical illness was observed between 7 and 19 days postinfection (dpi), with development in the animals of anorexia, weight loss, and depression (characterized by slumped, collapsed body posture and lack of responsiveness to the environmental triggers). These clinical signs progressed for several hours and were associated with hyperthermia and an acute respiratory syndrome characterized by dyspnea and hyperventilation. During the course of the disease, the animals became more obtunded and had uncoordinated motor movements, ending, in some instances, with a loss of consciousness and coma (Table 1). Although clinical signs were seen in monkeys infected intranasally and intravenously, the disease lasted longer in

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DOI: 10.3201/eid1603.091346

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Table 1. Clinical course of NiV infection in 12 squirrel monkeys*

Monkey	Mode of infection	Dose, PFU/mL	Day of 1st symptoms	Duration of clinical state	Day of euthanasia	Clinical state at euthanasia	Clinical signs
A†	IV	10^3	–	–	3	Well	None
B	IV	10^3	10	3	12†	Moribund	Uncoordinated motor movements, prostration and coma
C	IV	10^3	19	3	21‡	Moribund	Uncoordinated motor movements, prostration, and coma
D†	IV	10^7	–	–	3	Well	None
E	IV	10^7	7	2	8†	Moribund	Uncoordinated motor movements, prostration, and coma
F	IV	10^7	14	3	52	Recovered/well	Anorexia, depression
G†	IN	10^3	–	–	4	Well	None
H	IN	10^3	–	–	52	Well	None
I	IN	10^3	8	3	56	Recovered/well	Anorexia, seizure
J†	IN	10^7	–	–	4	Well	None
K	IN	10^7			17		Septic shock not correlated with NiV infection
L	IN	10^7	10	7	56	Recovered/well	Anorexia, seizure, edema of eyes

*IV, intravenous; IN, intranasal; NiV, Nipah virus.
†Early systematic euthanasia.
‡Death caused by Nipah virus infection.

intranasally infected animals (7 days) than in intravenously infected monkeys (2–3 days). With the latter, death was observed in 3 of 4 animals in which the disease was allowed to proceed. Clinical signs of illness for intranasally infected monkeys were milder and seen only in 2 of 4 animals before recovery after 3–7 days of illness. Clinical signs observed in monkeys appear to be similar to those reported for human infection, including involvement of neurologic and respiratory systems. In addition, the incubation period for the acute human infection in Malaysia was estimated to be from a few days to 2 weeks, total duration of illness ranged 2–34 days, and the rate of subclinical infection was ≈25% (*6*,*14*). It is possible that the inclusion of more animals in the study would have given higher heterogeneity in the course of disease, as seen in humans. Intravenous infection was much more efficient than the intranasal route in monkeys, probably because of a better delivery of the virus to different tissues.

NiV-specific RNA was detected in various organs only in intravenously infected animals (online Appendix Table, www.cdc.gov/EID/content/16/3/507-appT.htm), demonstrating a differential virus spread, depending on time after infection and virus dose. Early detection after infection (3 dpi) was possible only in animal D, which was infected with a high

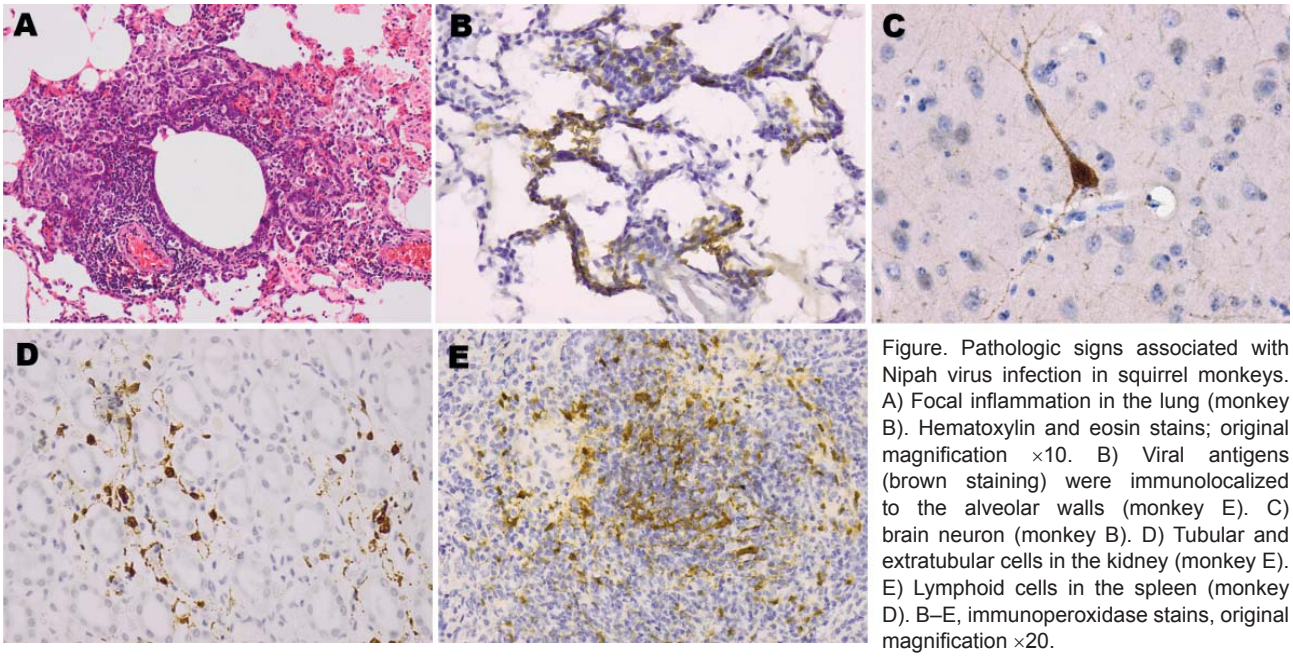

Figure. Pathologic signs associated with Nipah virus infection in squirrel monkeys. A) Focal inflammation in the lung (monkey B). Hematoxylin and eosin stains; original magnification ×10. B) Viral antigens (brown staining) were immunolocalized to the alveolar walls (monkey E). C) brain neuron (monkey B). D) Tubular and extratubular cells in the kidney (monkey E). E) Lymphoid cells in the spleen (monkey D). B–E, immunoperoxidase stains, original magnification ×20.

dose of NiV. Animal F, which recovered from the disease, although positive for NiV (by RT-PCR) in the PBMC sample 2 dpi, was negative after necropsy on day 52, when virus was probably eliminated from the monkey. Detection of viral RNA in different tissues (liver, brain, spleen, kidney, lung, lymph nodes) early after infection suggests a rapid propagation of NiV and tropism for various tissues. Viral RNA was found in PBMC taken at different time points after infection, suggesting the role of these cells in viral propagation in the monkey. In contrast to what has been observed in hamsters (*9*), viral RNA was not detected in any urine samples from analyzed animals, thus excluding urine as a possible mode of virus dissemination in this species.

Monkeys showed mild histologic lesions, including the inflammation most obvious in the lung parenchyma (Figure, panel A). In contrast to human infection, vasculitis and brain abnormalities were much less evident. However, immunohistochemistry showed viral antigens immunolocalized to the brain, lung, spleen, and kidney extravascular parenchyma, thus confirming viral infection in these organs (Figure, panels B–E).

NiV-specific immunoglobulin (Ig) M responses were observed starting from 8 dpi for all monkeys except in groups H and I (Table 2). This finding suggests that 10^3 PFU of NiV delivered intranasally was probably insufficient to induce infection in monkeys. Although NiV-specific anti-

Table 2. Detection of anti–Nipah virus antibodies in 12 squirrel monkeys by ELISA and seroneutralization assay*

Monkey	Mode of infection†	Serology‡	Day postinfection							
			2	3 or 4	8 or 9	12	17	30	37	52 or 56
A§	IV 10^3	IgM	–	Neg						
	IV 10^3	IgG	–	Neg						
	IV 10^3	Neutralization	–	Neg						
B¶	IV10^3	IgM	Neg	–	Neg	0.396				
	IV10^3	IgG	Neg	–	Neg	Neg				
	IV10^3	Neutralization	Neg	–	Neg	Neg				
C¶	IV10^3	IgM	Neg	–	0.664	–	–			
	IV10^3	IgG	Neg	–	Neg	–	–			
	IV10^3	Neutralization	Neg	–	Neg	–	–			
D§	IV 10^7	IgM	–	Neg						
	IV 10^7	IgG	–	Neg						
	IV 10^7	Neutralization	–	Neg						
E¶	IV 10^7	IgM	Neg	–	1.343					
	IV 10^7	IgG	Neg	–	0.562					
	IV 10^7	Neutralization	Neg	–	40					
F	IV 10^7	IgM	Neg	–	1.550	–	–	0.374	–	0.175
	IV 10^7	IgG	Neg	–	0.369	–	–	2.867	–	3.023
	IV 10^7	Neutralization	Neg	–	80	–	–	>1,280	–	>1,280
G§	IN 10^3	IgM	Neg	Neg						
	IN 10^3	IgG	Neg	Neg						
	IN 10^3	Neutralization	Neg	Neg						
H	IN 10^3	IgM	–	–	Neg	–	–	Neg	–	Neg
	IN 10^3	IgG	–	–	Neg	–	–	Neg	–	Neg
	IN 10^3	Neutralization	–	–	Neg	–	–	Neg	–	Neg
I	IN 10^3	IgM	–	–	Neg	–	–	Neg	Neg	Neg
	IN 10^3	IgG	–	–	Neg	–	–	Neg	Neg	Neg
	IN 10^3	Neutralization	–	–	Neg	–	–	Neg	Neg	Neg
J§	IN 10^7	IgM	–	Neg						
	IN 10^7	IgG	–	Neg						
	IN 10^7	Neutralization	–	Neg						
K	IN 10^7	IgM	Neg	–	–	–	0.286			
	IN 10^7	IgG	Neg	–	–	–	1.757			
	IN 10^7	Neutralization	Neg	–	–	–	80			
L	IN 10^7	IgM	–	–	0.375			0.248		Neg
	IN 10^7	IgG	–	–	Neg			2.078		2.308
	IN 10^7	Neutralization	–	–	Neg			320		320

*IV, intravenous; Ig, immunoglobulin; neg, negative; IN, intranasal.
†Mode of infection and delivered viral dose (in PFU).
‡IgG and IgM antibodies were determined by ELISA and results are presented as absorbance readings. Neutralization titers are expressed as reciprocal values of 2-fold serum dilutions required to completely inhibit cytopathic effect of 25 PFU of NiV on Vero cells.
§Early systemic euthanasia: 3 days postinfection (dpi) (A and D) or 4 dpi (G and J).
¶Death caused by NiV infection: B, 12 dpi; C, 21 dpi; E, 8 dpi.

bodies were detected by ELISA in animals dying from the infection, sufficient titers of neutralizing antibodies did not develop in these monkeys and they were therefore not protected. These findings suggest the protective role of high neutralization titers in NiV infection. Our results agree with other studies of NiV infection that reported most human patients with fatal NiV infection had IgG and IgM in their serum and cerebrospinal fluid (*6*,*15*); neutralization titers were not analyzed in those studies.

Our results suggest some similarities of NiV pathogenesis in humans and squirrel monkeys, including development of clinical signs, progression of infection, and humoral immune response. We conclude that the squirrel monkey can be used as an animal model for experimental studies of NiV infection, and these results pave the way for further study.

Acknowledgments

We thank BioSafety team members from BSL-4 Laboratory "Jean Mérieux" for their assistance.

The work was supported by Institut Pasteur, Institut national de santé et de la recherché médicale, ANR 2005 program "Microbologie, infections et immunités" (ANR-05-MIIM-017-02), and the government of Malaysia (IRPA 06-02-03-0000-PR0060/04).

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Q Fever in Greenland

Anders Koch, Claus Bo Svendsen, Jens Jørgen Christensen, Henning Bundgaard, Lars Vindfeld, Claus Bohn Christiansen, Michael Kemp, and Steen Villumsen

We report a patient with Q fever endocarditis in a settlement in eastern Greenland (Isortoq, Ammassalik area). Likely animal sources include sled dogs and seals. Q fever may be underdiagnosed in Arctic areas but may also represent an emerging infection.

Q fever is a zoonosis caused by the small intracellular bacterium *Coxiella burnetii*. Main reservoirs for this bacterium are cattle, goats, and sheep, although a wide range of animals may be infected (*1*,*2*). *C. burnetii* can survive in a spore-like form under harsh conditions (*2*).

In animals, *C. burnetii* infection is often latent; the bacteria may be persistently shed into the environment, especially at the time of giving birth (*2*). In humans, most acute cases result in asymptomatic or mild influenza-like disease; severe disease develops in a few patients. Primary manifestations include pneumonia, hepatitis, and fever of unknown origin.

Q fever has been described in >59 countries (*1*) but not in Arctic areas. We report a patient with Q fever in Greenland.

The Patient

The patient, a 40-year-old man, who resided in Greenland all his life, lived in Isortoq (population 100), a small settlement in the Ammassalik area (population 3,000) of eastern Greenland (Figure). He had worked as a hunter and a sanitation worker (garbage collector). The Ammassalik area includes the main town of Tasiilaq and 5 settlements. Isortoq is located on an island off the coast of Greenland. Access is by helicopter, boat during the summer, and dog sleds and snowmobiles during the winter. The main occupation is hunting, especially of seals, which are consumed locally. All other food is imported though Tasiilaq. All imported meat is frozen, and only ultra-high-temperature–pasteurized milk is available. Terrestrial mammals in the area include sled dogs, polar foxes, and a few domesticated cats. Sea mammals include seals and walruses. Polar bears are abundant throughout eastern Greenland; the nearest sheep, horses, and musk oxen are >1,000 km away. There are no cows and goats in Greenland.

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DOI: 10.3201/eid1603.091220

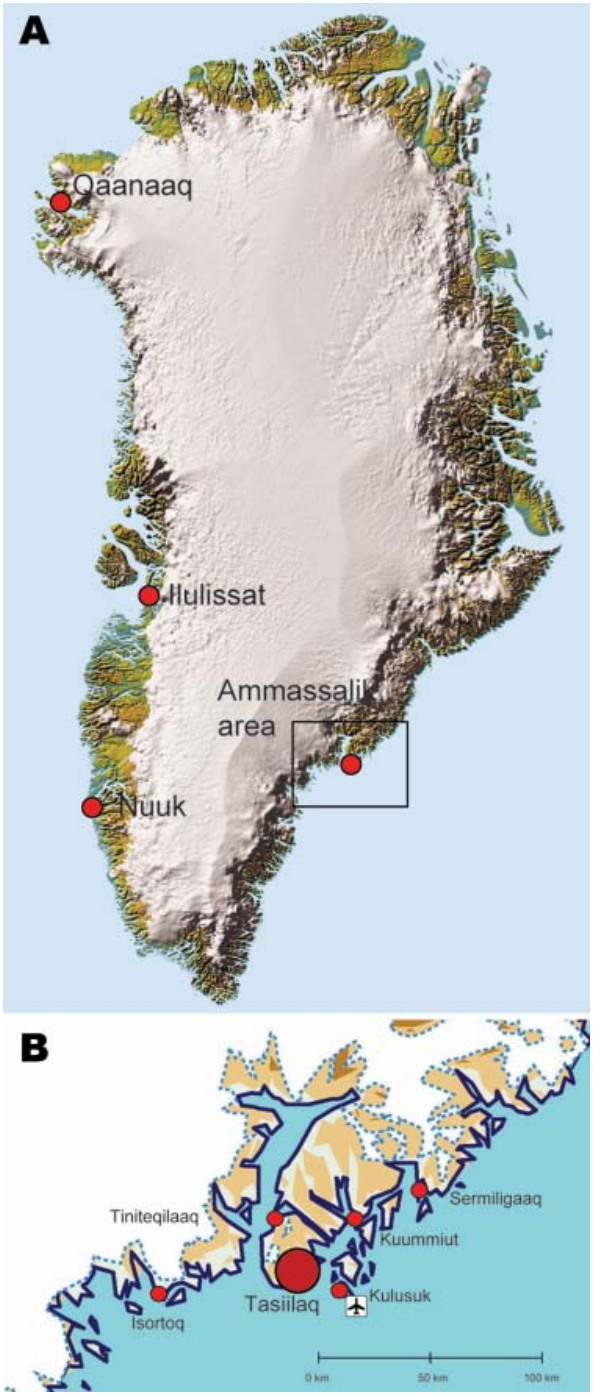

Figure. A) Ammassalik area (box) in Greenland. B) Main towns in the Ammassalik area. Red circles show the main town of Tasiilaq and 5 settlements. Location of the airport is indicated. Reprinted with permission of the National Survey and Cadastre [Kort og Matrikelstyrelsen], Danish Ministry of the Environment, Copenhagen, Denmark.

In December 2007, the patient came to Tasiilaq Health Center with dyspnea, chest pains, and fever that had lasted 2 months. In 2001, because he had had rheumatic fever during childhood, he had biological aortic and mitral valves implanted. As a result, he was at increased risk for Q fever endocarditis (*3*). However, he was healthy and had not taken any medications during the preoperative period.

In January 2008, the patient was transferred to University Hospital Rigshospitalet in Copenhagen, Denmark. Clinical findings included low-grade fever, cardiac insufficiency with peripheral edema, hepatosplenomegaly, and 20% half-moon nephritis; a transesophageal echocardiograph did not show signs of endocarditis. Repeated blood cultures were negative for bacteria.

In May 2008, an echocardiograph showed aorta and mitral valve vegetations and stenoses. Subsequent surgery showed massive endocarditis. His biological valves were replaced with mechanical valves. Recovery was uneventful, signs of heart failure disappeared, and laboratory test results and cardiac function gradually returned to reference levels.

A sample from his resected cardiac valves was subjected to routine partial 16S rRNA PCR and DNA sequencing (*4*). A BLAST search in the National Center for Biotechnology Information (Bethesda, MD, USA) database showed 502 of 502 bp to be identical with those of *C. burnetii*. Identification was confirmed by PCR specific for the *C. burnetii* transposase (IS*1111*a) gene by using primers CoxiellaF1x (5′-GTA TCG GAC GTT TAT GGG GAT GGG TAT CC-3′) and CoxiellaR1 (5′-CAC CAC GCG CCA TCG TGA GTC-3′). PCR conditions were 10 cycles at 95°C for 30 s and 75°C–65°C for 60 s and 40 cycles at 95°C for 30 s and 65°C for 60 s.

Subsequent culture in Vero cells was positive after incubation for 30 days, and results were confirmed by indirect immunofluorescent assay with *C. burnetii* phase II–specific antibodies (Australian Rickettsial Reference Laboratory, Geelong, Victoria, Australia). A nearly full-length sequence of the 16S rRNA gene was obtained by DNA sequencing of culture material and yielded 1,321- of 1,321-bp sequence homology with *C. burnetii*, including the original sequence obtained directly from the valve. This DNA sequence has been submitted to GenBank (accession no. 1188993 FJ787329) as the Ammassalik strain.

Blood obtained 27 days before surgery was positive by *C. burnetii*–specific PCR. A sample contained high levels of *C. burnetii*–specific antibodies by immunofluorescent assay (Focus Diagnostics, Cypress, CA, USA): immunoglobulin (Ig) M titer phase I, 16,000; IgM phase II, 16,000; IgG phase I, 512,000; and IgG phase II, 1,024,000.

In April 2004, the patient had participated in a population-based screening in the Ammassalik area for antibodies against *Trichinella* spp. and *Anisachis* spp. (*5*). His serum was stored at –80°C at Statens Serum Institut and tested for antibodies against *C. burnetii* after illness was reported in 2008; results for IgM and IgG were negative. Except for his cardiac surgery in 2001 in Copenhagen, the patient had never been outside the Ammassalik area.

Conclusions

The patient was likely infected in the Ammassalik area in 2007, rather than during or before 2004. His stored serum sample was negative for *C. burnetii* in 2004. The lack of domesticated ruminants may be the reason why Q fever has not been described in Arctic areas. Although *C. burnetii* has not been isolated from Arctic animals, some musk oxen in northern Quebec and reindeer in Arctic Russia (Nenet region) have been found to be positive for IgG against *C. burnetii* (*6,7*). Likewise, <0.6% of Inuits from Nunavik, 15% of trappers, and 18% of Cree hunters from interior regions of southern Quebec have been found to be positive for IgG against *C. burnetii* (*8–10*).

In the absence of raw milk products, animals represent the most likely source of infection in eastern Greenland. *C. burnetii* has been found in dogs, cats, birds (*2*), seals (*11*), and bears (*12,13*) in other regions. For our patient, we cannot rule out the possibility that infection may have been caused by a domestic cat that may have traveled with its owner to a region endemic for Q fever or by migratory birds. However, because endocarditis is a rare manifestation of Q fever and affects <0.5% of all case-patients (*2*), *C. burnetii* may be endemic to the Arctic area. The most likely animal reservoirs would be sled dogs or seals because a herd of a certain size is necessary to sustain infection in an animal population. Sled dogs are mostly kept chained in groups, and bacteria may spread from infected placentas to other dogs and humans in the vicinity. Seals are abundant in the Ammassalik area and represent a major human food source. Harbor and hooded seals form colonies at time of giving birth, when infection is most likely to spread (*11*). Polar foxes and bears are less likely reservoirs because their populations are less dense (*2*).

Whether Q fever is an underdiagnosed or emerging disease in the Arctic area is unknown. Because many cases are asymptomatic and laboratory facilities in Greenland are few, this disease, although rare, may be underdiagnosed in this country. However, Q fever may also be an emerging infection, possibly related to climate changes, as seen elsewhere in the Arctic area (*14*).

Serologic studies of persons in the Arctic region, including Greenland, may shed light on these issues. Possible animal reservoirs may be identified by serologic studies of wildlife. In addition, health authorities in the Arctic area need to be aware of *C. burnetii* as a possible infectious agent.

Acknowledgments

We thank the patient for permission to publish his medical history; staff members at the hospitals to which the patient had been referred, particularly Hans Christian Florian Sørensen, Karin Ladefoged, Søren Thybo, Jørgen Kurtzhals, and Karen A. Krogfelt, for assistance; Lone Nukaaraq Møller for permission to use the stored serum sample from 2004; Kathrine Friholm Villumsen for information about behavior of Arctic animals; and Benoit Levesque for information about Q fever in the Arctic region of Canada.

S.V. is a member of MedVetNet, Centre of Excellence, which was supported by grant FP7.

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Banna Virus, China, 1987–2007

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Banna viruses (BAVs) have been isolated from pigs, cattle, ticks, mosquitoes, and human encephalitis patients. We isolated and analyzed 20 BAVs newly isolated in China; this finding extends the distribution of BAVs from tropical zone to north temperate climates and demonstrate regional variations in BAV phylogeny and mosquito species possibly involved in BAV transmission.

Banna virus (BAV), the prototype species of genus *Seadornavirus* within the family *Reoviridae*, has a genome composed of 12 segments of double-stranded RNA (*1*). BAV was initially isolated from persons with encephalitis and fever in Xishuangbanna, Yunnan Province, People's Republic of China, in 1987 (*2*). Since then, BAV isolates have been obtained from pigs, cattle, and ticks in China (*3*,*4*) and from mosquitoes in Indonesia, China, and Vietnam. (*5*–*7*). BAV is a BioSafety Level 3 arboviral agent that is pathogenic to humans and may well be an emerging pathogen or undiagnosed cause of human viral encephalitis in some areas (*1*). Our objective was to describe new BAV isolates from China and to define the geographic distribution and the phylogenetic relationships of these isolates with reference to the previously described isolates.

The Study

In this study, 20 new BAV isolates were obtained from mosquitoes collected from July through September during 2006 to 2007 at sites in Gansu Province (latitude 32°–35°N, 104°–107°E), Liaoning Province (39°–41°N, 123°–125°E), Shanxi Province (37°–38°N, 111°–113°E), and Inner Mongolia Province (41°–43°N, 121°–123°E) (Table, Figure 1). Mosquito samples were collected by using 12 V, 200 mA mosquito-trapping lamps (Wuhan Lucky Star Environmental Protection Tech Co., Ltd., Hubei, China) and by collecting mosquitoes from 8:00 PM to 11:00 PM at nearby cow barns, a piggery, and fish pond sites where human activity was frequent. Mosquitoes were put into a –20°C freezer for 30 min and then were rapidly sorted into pools of 50 to 100 specimens according to species. The pools were put into labeled tubes and stored in liquid nitrogen.

Viruses were isolated and BAV isolates were identified using described procedures (*8*). Trizol reagent category no. 10296-028 (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. cDNA was prepared by using Ready-to-Go You-Prime First-Strand Beads Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's protocol. An 850-bp gene fragment from the 12th segment, which codes for the double-stranded RNA binding protein, was amplified from the cDNA of the BAV isolates by using previously published primers (*9*). PCR products were recovered by using purification kits (QIAGEN, Valencia, CA, USA), and then were inserted into pGEM-T easy vector (Promega, Madison, WI, USA). The insert sequence was determined by using M13 universal primers and an ABI Prism 3730 sequence analyzer (ABI, Shirley, NY, USA).

The genomic sequences of the 12th segment for the 20 new BAV strains were determined (GenBank accession nos. GQ331954–GQ331973). Phylogenetic trees were constructed from the amplified region of the 12th segment sequence by using the molecular evolutionary genetics analysis (MEGA) version 4 software (www.megasoftware.net) from aligned nucleotide sequences. We used neighbor-joining algorithms with 1,000 replicates for bootstrap support of tree groupings.

In this study, 38 BAV strains isolated during 1987–2007 were analyzed, which included 30 strains isolated in China (including 20 new BAV isolates first reported in this study and 10 previously described isolates from China (*8*,*10*–*12*), 3 strains from Indonesia, and 5 strains from Vietnam) (Table). Initial BAVs were isolated from Indonesia and Yunnan Province of China, which belong to tropical and subtropical zones (*2*,*5*).The new BAV isolates in our study were observed in Gansu, Shanxi, Liaoning, and Inner Mongolia provinces of China (northern China), which belong to the northern temperate zone. These strains represent a geographic distribution ranging from near the equator to latitude 45°N, extending from the tropical zone to the northern temperate zone (Figure 1). These data show that the distribution of BAVs is not limited to Southeast Asia but that it extends into northeast Asia as well.

Before our study, BAV had been isolated from 7 mosquito species in 2 genera (*Culex tritaeniorhynchus, Cx. pipiens pallens, Cx. annulus, Cx. pseudovishnui, Cx. modestus, Anopheles sinensis,* and *Aedes vagus*). To this list we now add 3 species in the genus *Aedes* (*Ae. albopictus, Ae. vexans,* and *Ae. dorsalis* (Table), which are widely distributed in China and elsewhere.

Phylogenetic analysis based on the complete coding sequence (624 nt) of the 12th segment of the BAV genome

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DOI: 10.3201/eid1603.091160

indicated that the BAV isolates evaluated in this study could be divided into 2 phylogenetically different groups (Figure 2). Isolates from China and Vietnam are included in group A, and the strains from Indonesia are in group B. Group A could be further divided into 2 subgroups, A1 and A2. Subgroup A1 includes 4 independent clades that group according to the location of collection and represent viruses from northern China (Gansu, Shanxi, and Liaoning Provinces) as well as the Vietnam isolates. Subgroup A2 includes isolates mainly from southern China (Yunnan Province) and Vietnam, which is contiguous with Yunnan Province of China, as well as 2 isolates from northern China (BJ95-75/Beijing, and NM0706/Inner Mongolia) (Figure 1).

Conclusions

Our results demonstrate that BAV strains are distributed from the tropics of Southeast Asia to the northern temperate regions of China. These observations suggest that the distribution of BAV is wider than previously recognized and may be increasing. Consistent with previous observations (*9*), we report that BAV isolates from China cluster in group A and separate into subgroups mainly according to the geographic origin of the isolate; subgroup A1 is found in the north and subgroup A2 in the south. However, 2 isolates from northern China grouped in subgroup A2 (south), and 3 isolates from Vietnam grouped in subgroup A1 (north).

Considering that group A isolates are geographically located across the monsoon climate zone, where south-to-

Table. Distribution of Banna viruses in regions and vectors, China

Region	Country	Province	Strain	Origin	Date of collection	Vector	Accession no.	Reference
Temperate zone	China	Gansu	GS07-KD12	Cow barn	2007 Aug	*Anopheles sinensis*	GQ331954	This study
			GS07-KD15	Cow barn	2007 Aug	*Culex tritaeniorhynchus*	GQ331955	This study
			GS07-KD16	Cow barn	2007 Aug	*Cx. pipiens pallens*	GQ331956	This study
			GS07-KD18	Cow barn	2007 Aug	*An. sinensis*	GQ331957	This study
			GS07-KD27	Piggery	2007 Aug	*Cx. tritaeniorhychus*	GQ331958	This study
			GS07-KD29	Piggery	2007 Aug	*Aedes albopictus*	GQ331959	This study
			GS07-KD30	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331960	This study
			GS07-KD32	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331961	This study
			GS07-KD38	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331962	This study
			GS-KD42-2	Piggery	2006 Aug	*Cx. tritaeniorhychus*	FJ160414	(*8*)
		Shanxi	SX0765	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331963	This study
			SX0766	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331964	This study
			SX0767	Piggery	2007 Aug	*Ae. vexans*	GQ331965	This study
			SX0771	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331966	This study
			SX0789	Piggery	2007 Aug	*Ae. dorsalis*	GQ331967	This study
			SX0790	Piggery	2007 Aug	*Ae. vexans*	GQ331968	This study
			SX0793	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331969	This study
			SX0794	Piggery	2007 Aug	*Ae. dorsalis*	GQ331970	This study
			SX0795	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331971	This study
			SX0796	Piggery	2007 Aug	*Cx. pipiens pallens*	GQ331972	This study
		Inner Mongolia	NM0706	Fishpond	2007 Aug	*Cx. modestus*	GQ331973	This study
		Liaoning	LN0684	Piggery	2006 Aug	*An. sinensis*	FJ217989	(*11*)
			LN0688	Piggery	2006 Aug	*An. sinensis*	FJ217990	(*11*)
			LN0689	Piggery	2006 Aug	*An. sinensis*	FJ217991	(*11*)
		Beijing	BJ95-75	Unknown	1995	Unidentified mosquito	AY568289	(*12*)
Subtropical zone	China	Yunnan	YN-6	Unknown	2001	Unidentified mosquito	AY568290	(*12*)
			YN0556	Unknown	2005 Jul	*Cx. tritaeniorhychus*	FJ161966	(*10*)
			YN0558	Unknown	2005 Jul	*Cx. tritaeniorhychus*	FJ161964	(*10*)
			YN0659	Unknown	2005 Jul	*An. sinensis*	FJ161965	(*10*)
	Vietnam	Quang Binh	02VN180b	Unknown	2002 Aug	*Cx. tritaeniorhychus*	EU265727	(*7*)
			02VN178b	Unknown	2002 Aug	*Cx. tritaeniorhychus*	EU265715	(*7*)
			02VN018b	Unknown	2002 Mar	*Cx. annulus*	EU265694	(*7*)
		Ha Tay	02VN009b	Unknown	2002 Jan	*Cx. annulus*	EU265682	(*7*)
			02VN078b	Unknown	2002 May	*Cx. tritaeniorhychus*	EU265705	(*7*)
Tropical zone	Indonesia	Java	JKT-6423	Unknown	1980	*Cx. pseudovishnui*	NC004198	(*5*)
			JKT-6969	Unknown	1981	*Ae. vagus*	AF052008	(*5*)
			JKT-7043	Unknown	1981	*Cx. pipiens pallens*	AF052024	(*5*)

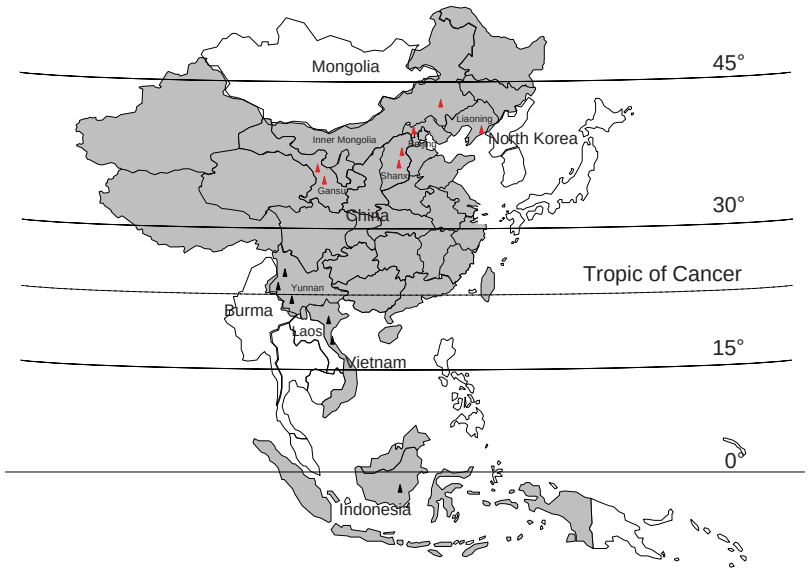

Figure 1. Location of new Banna viruses (BAVs) isolated in China (red triangles) and previously reported BAV isolation sites (black triangles). Countries reporting isolation of BAV are shaded. The names of the countries that are contiguous with BAV isolation sites are labeled. BAV distribution sites in Indonesia, Vietnam, and part of China are located in tropical zones, which lie predominantly between the Tropic of Cancer and the equator. Most BAV distribution sites in China in the area from the Tropic of Cancer to latitude 45°N belong to the northern temperate zone.

north winds are common during summer (*13*), BAV could be transferred in infected mosquitoes during this period by the prevailing winds that move from Southeast Asia to east Asia. In addition, bird migration, has been associated with the movement of other pathogens, and migration of infected birds through the east Asia–Australasia flyway (*13*), which traverses the region, may also account for this association. However, the transmission dynamics of BAVs are not well known. Further study is required to determine if winds and birds are involved in dispersal of the virus.

Our observations suggest that the public health impact of BAV may be underestimated. BAV appears to be ac-

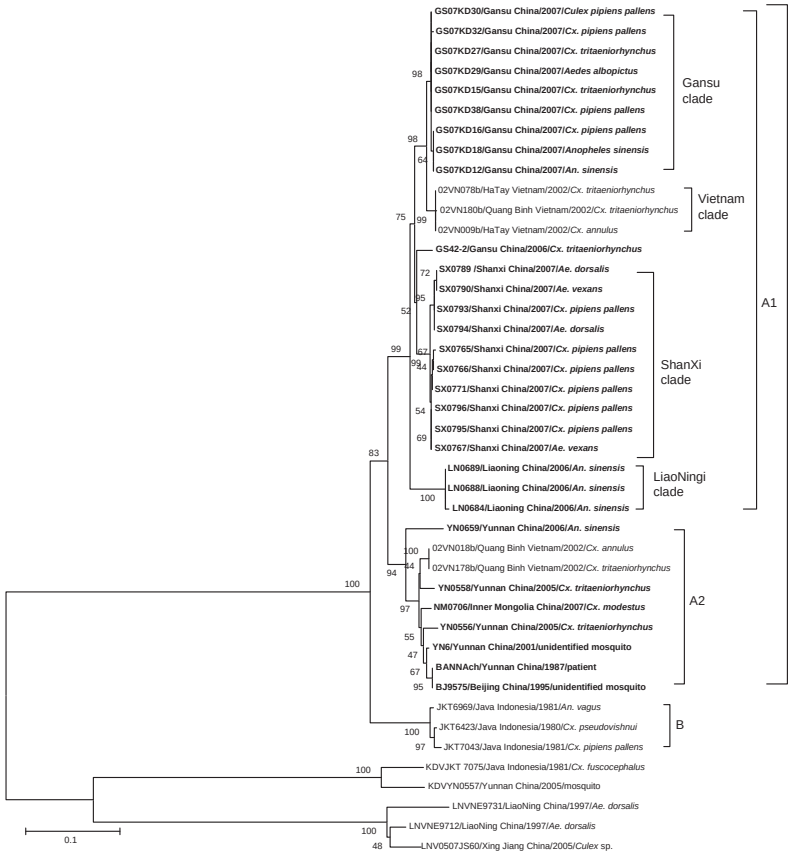

Figure 2. Phylogenetic analysis based on the complete coding sequence of the 12th segment of Banna viruses (BAVs) currently isolated. Phylogenetic analyses were performed by the neighbor-joining method using MEGA version 4 software (www.megasoftware.net). Bootstrap probabilities of each node were calculated with 1,000 replicates. The tree was rooted by using Kadipiro virus and Liaoning virus as the outgroup viruses. Scale bars indicate a genetic distance of 0.1-nt substitutions per site. Isolates obtained in China are in **boldface**. Viruses were identified by using the nomenclature of virus strain/country/year of isolation/origin.

tively circulating in areas where Japanese encephalitis virus (JEV) is endemic (*14*) and where *C. tritaeniorhynchus*, which is the main vector of JEV, is active. This mosquito also appears to be a common vector of BAV. The clinical symptoms of disease caused by the 2 viruses is similar, and BAV cases may be undetected during a JE outbreak. It has been reported that ≈14% of clinically diagnosed JE cases are BAV immunoglobulin (Ig) M positive (*15*), indicating that BAV epidemics may have occurred but have been clinically misdiagnosed as Japanese encephalitis. The apparent active transmission of BAV over a large geographic area, genetic variation between geographic regions, and the potential to cause severe disease underscore the need for additional surveillance, further characterization, and improved diagnostic systems worldwide.

Acknowledgments

We thank Roger S. Nasci for consultation and assistance in the preparation and writing of this manuscript.

This work was supported by grants from the Ministry of Science and Technology, China (2003BA712A08-01; 2008ZX10004-008); Chinese CDC–US Centers for Disease Control and Prevention Cooperative Agreement U19-GH000004; Development Grant of State Key Laboratory for Infectious Disease Prevention and Control (2008SKLID105); and Programme of Research Advance, China-France, B-06-04.

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Bluetongue Virus Serotypes 1 and 4 in Red Deer, Spain

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We studied the potential of red deer as bluetongue maintenance hosts and sentinels. Deer maintained detectable bluetongue virus (BTV) serotype 4 RNA for 1 year after the virus was cleared from livestock. However, the virus was not transmitted to yearlings. BTV serotype 1 RNA was detected in red deer immediately after its first detection in cattle.

Bluetongue (BT) is a vector-borne disease caused by a virus belonging to the genus *Orbivirus*, with 24 known serotypes (*1*). Since 2000, four of these seroptypes have been found in Spain on 5 occasions: 1) Bluetongue virus serotype 2 (BTV-2) was detected in 2000 in the Balearic Islands, 2) BTV-4 was detected in 2003 in the Balearic Islands, 3) a different BTV-4 strain was detected in 2004 in southern Spain, 4) BTV-1 was detected for the first time in 2007 in Spain, and 5) BTV-8 was detected in 2008 in Spain after it entered through the border with France. In livestock, BTV-4 was detected for the last time in November 2006, and the country was declared free of BTV-4 in March 2009 by the European Union Standing Committee on the Food Chain and Animal Health (http://rasve.mapa.es/Publica/Noticias/Ficheros/Informe libre serotipo 4 final.pdf). Currently, all of Spain is considered a restriction zone for BTV-1 and -8.

Sheep are considered the most vulnerable species for BT, but other ruminants are known to play a major role in BT epidemiology. The role of wild ruminants in the spreading and persistence of the virus has only begun to be elucidated. Several studies have reported the presence of either BTV antibodies (*2*,*3*) or the virus (*4*) in red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), mouflons (*Ovis aries*), and several other wild bovids and cervids (*2*,*5*). The presence of BTV and BTV-specific antibodies in wild species underscores the role of these species, because, except for mouflons (*4*), European wild ruminants generally are asymptomatic hosts. The highest peak of stress occurs during the mating period (August–September in Spain), which is also the period of maximal activity for *Culicoides imicola* mosquitoes. Therefore, all of these facts, together with the capability of wild ruminants to overcome BT infection and their free-range life, make deer suitable for BTV maintenance. We hypothesize that 1) BTV RNA would be detectable in red deer even after its control in livestock by vaccination, and 2) the virus or specific antibodies would be detected in red deer early after its detection in livestock.

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DOI: 10.3201/eid1603.090626

The Study

The study site was a deer farm with ≈900 hinds, including 550 adult hinds and 350 yearling hinds. This farm is located in the Los Alcornocales Natural Park in the Cádiz Province (Andalucía, southern Spain; 36°17′N, 5°47′W), an area near the sea that is <500 m above sea level. Abundant wild red deer and moderate densities of roe deer (*Capreolus capreolus*) are present in the area.

Blood samples were collected by cervical puncture from 510 living farmed red deer, placed in sterile tubes containing EDTA, and frozen at –20°C. Samples from adult deer hinds (n = 160) were obtained on July 12 and 13, 2007; yearling stags (n = 350) were sampled on August 28, 2007.

We tested 200 serum samples by using a competitive viral protein 7 (VP7) ELISA (Institute Pourquier, Montpellier, France). The samples were analyzed in duplicate according to the manufacturer's instructions.

After RNA extraction from 510 red deer blood samples, RNA was analyzed by using 4 reverse transcription–PCRs (RT-PCRs): 1) a group-specific RT-PCR detecting a conserved region within the BTV nonstructural protein (NS) 1 segment (*6*); 2) a BTV-1 serotype-specific RT-PCR (*7*); 3) a BTV-4 serotype-specific assay (*8*); and 4) a group-specific RT-PCR that detects epizootic hemorrhagic disease (EHD) (*9*). BTV-4 PCR was performed as a 1-step real-time RT-PCR, and BTV-1, EHD, and the group-specific assays were conducted as gel-based, 1-step RT-PCRs. Prevalence of BT antibodies and BTV-1 and BTV-4 RNA and confidence intervals for prevalence (binomial exact, Clopper-Pearson) were calculated by using Quatitative Parasitology 3.0 software (*10*).

Of the analyzed serum samples, 57.60% showed positive results in the ELISA. The prevalence of BTV antibodies was high; 92.45% of the adults were positive. All yearling deer were ELISA negative except for 3 doubtful samples; all of them had negative results in the BTV, BTV-1 and BTV-4 RT-PCR assays (Figure 1).

Of the adult deer, 25% showed positive results in the BTV group-specific PCR. Positive samples were sequenced to confirm the presence of BTV nucleic acid and further analyzed for the identification of the serotype. Six RNA samples from adult deer were positive for the BTV-4–specific RT-PCR, and their sequences were confirmed by using

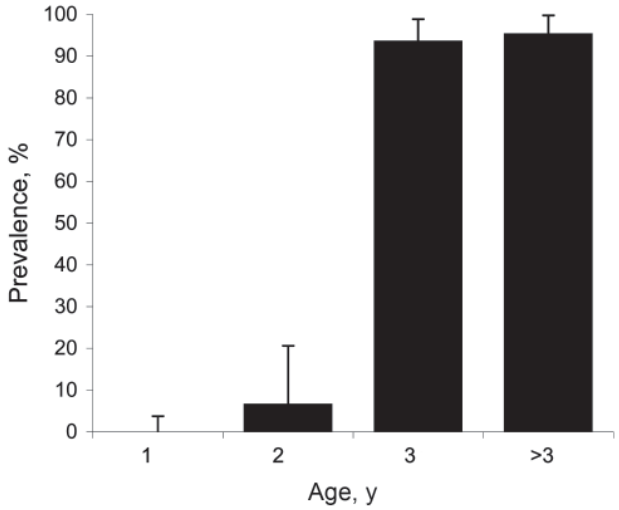

Figure 1. Results of ELISA to detect bluetongue virus (BTV) viral protein 7 in 200 serum samples collected from red deer, Spain. Results from yearlings were negative; results from adults showed an age-increasing trend of contact with BTV. Bars represent 95% confidence intervals for prevalence (binomial exact, Clopper-Pearson).

BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). None of the samples from adult deer were positive either for BTV-1-specific or EHD-specific RT-PCRs. Yearlings, however, showed a different pattern of results: 16.33% animals showed positive results in the group-specific and the BTV-1–specific RT-PCRs. No yearling samples were positive by the BTV-4 specific RT-PCR.

No visible clinical signs were noticed, and no deaths occurred. This result suggests that, although adult deer maintained circulation of BTV-4 RNA, this serotype did not infect the yearlings despite the presence of the vector and the optimal conditions for infection in the study area. Surprisingly, several animals were positive to the EHD-specific assay. However, when the PCR products were purified and sequenced, none of the obtained sequences showed homology with published EHD sequences. These results support those found by Agüero et al. (*11*), in which BTV-1–positive samples cross-reacted with the available EHD primers. The amplified PCR product obtained had approximately the same size as the PCR product expected for EHD, thus giving a false-positive result.

Conclusions

Our results agree with what was found in livestock during surveillance programs: adult animals had probably been in contact with BTV-4 during the outbreak that started in southern Spain in 2004. In contrast to the vaccinated domestic ruminants, deer were able to maintain BTV-4 RNA, thus confirming our initial hypothesis. However, detection of BTV RNA without concurrent virus isolation does not mean that deer are a long term reservoir host of BTV (*12*). Simultaneous evaluation of adjacent cohorts of domestic and wild ruminants by using the same virus detection assays will be required to unambiguously define the precise role of wildlife in the epidemiology of BTV infection.

Yearling deer were apparently infected with BTV-1, which has been present in Spain since 2007. When epidemiologic information about the study area was compared with the information for the deer samples analyzed, evidence was found supporting our results: adult deer were sampled on July 12, 2007, and yearlings were sampled August 20, 2007, i.e., 26 days after BTV-1 presence was confirmed at 60 km distance from the deer farm (www.oie.int/wahis/reports/en_imm_0000005799_20070726_123322.pdf) (Figure 2).

Thus, adult deer had been sampled when BTV-1 was not present in the country yet. In contrast, yearlings were already positive to BTV-1 only 26 days after this serotype was first reported in livestock in the same area. There are 2 explanations for this finding: 1) BTV-1 is a highly pathogenic serotype (*13*), causing high death rates in sheep, that may also cause high death rates in deer; and 2) deer and other wild ruminants may be highly susceptible to BTV infection, thus, making them good sentinels for this disease. However, BTV-1 was detected earlier among sentinel cattle than among deer.

Regarding EHD, despite the negative results obtained, lack of robust molecular tools for its detection is noteworthy. All available RT-PCRs are based on the sequences of EHD strains that have never been detected in the Mediterranean area.

Figure 2. Epidemiologic situation for bluetongue virus (BTV) in Spain, July–August 2007. The first BTV-1 case in Spain was reported in Tarifa (purple circle), only 60 km west from a deer farm where the samples were collected (blue diamond). Map source: Google Maps.

Acknowledgments

We thank José Antonio Ortiz (field veterinarian) and the laboratory technicians from the Animal Health Department Belén Rivera and Rocío Sánchez for involvement in and dedication to this study.

This study was funded by the Spanish Ministry of Natural, Rural and Marine Environment (RASVE 274/2007, and an agreement between Organismo Autónomo de Parques Nacionales (OAPN), Dirección General de Recursos Agricolas y Ganaderos (DGRAG), and Consejo Superior de Investigaciones Cientificas (CSIC). F.R.-F. is supported by a postdoctoral contract of the Instituto de Salud Carlos III of the Spanish government.

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Novel Spotted Fever Group Rickettsiosis, Brazil

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We report a clinical case of spotted fever group rickettsiosis acquired in São Paulo, Brazil. Definitive diagnosis was supported by seroconversion between acute-phase and convalescent-phase serum samples. Molecular analysis of skin samples indicated the agent was a novel spotted fever group strain closely related to *Rickettsia africae, R. parkeri,* and *R. sibirica*.

Rickettsia rickettsii is the etiologic agent of Rocky Mountain spotted fever (RMSF). During the past 2 decades, a clear reemergence of RMSF has been seen in southeastern Brazil, where ≈350 laboratory-confirmed cases (case-fatality rate ≈30%) have been reported (*1*). Most of these cases were confirmed solely by serologic-based techniques; specific identification of the *Rickettsia* species was not achieved. However, because these cases were clinically and epidemiologically compatible with RMSF, the agent was presumed to be *R. rickettsii* (*1*).

The occurrence of *R. parkeri* in Brazil has been restricted to ticks; human clinical infection has been reported in the United States, and possibly in Uruguay (*1*). Additionally, a few clinical cases caused by *R. felis* or *R. typhi* have been reported in southeastern Brazil during the 21st century (*2*,*3*). We report a clinical case of SFG rickettsiosis in a patient from southeastern Brazil. Molecular analysis of clinical samples showed that the patient was infected by a novel SFG strain.

Case Report

On May 2, 2009, a 66-year-old man was bitten by a tick on his lumbar region while walking on his ranch within an Atlantic rainforest area. Although his primary residence was in the urban area of Santo André within the São Paulo Metropolitan region (where he reported never having been bitten by ticks), he often visited his ranch in Barra do Una, a village within the Peruíbe Municipality, southern coastal region of the state of São Paulo (where he reported having been bitten by ticks several times). The area is within a large Atlantic rainforest reserve and is <50 m above sea level. The patient reported no travel to additional locations during the previous 3 months. Ten days after the tick bite (May 12, 2009), the patient reported the first episode of fever (≈39°C) and took acetaminophen. On May 15, 2009, he visited a doctor, who prescribed oral cephalexin (500 mg, 6×/6 h). On the next day, a macular rash appeared on his arms and legs, associated with muscle and joint pain. Fever was still present (39.5°C). On May 19, 2009, the patient had continuing fever (39.5°C), a macular rash (without itch) on his arms and legs, arthralgia, and myalgia on lower regions of the arms and legs and hands. The patient had an eschar on the lumbar region (Figure 1), exactly where he had removed an attached tick on May 2, 2009. He admitted that the tick remained attached to his skin for at least 20 hours until being removed and discarded.

Based on suspicion of rickettsial disease, blood samples were collected the same day, and doxycycline (100 mg, 12×/12 h) was prescribed for 10 days. Three days later (May 22, 2009), the patient returned to the laboratory where a new blood sample was collected, and a skin biopsy of the eschar was aseptically performed. The patient had not had a fever since May 20, 2009 (1 day after initiation of doxycycline therapy), but still had macular rash and joint and muscle pain. A third blood sample was collected 13 days later (June 4, 2009), and no clinical abnormalities were found.

Blood serum was tested by using an immunofluorescent antibody assay with antigens from 6 *Rickettsia* species that are present in Brazil: *R. rickettsii*, *R. parkeri, R. felis, R. amblyommii, R. rhipicephali,* and *R. bellii* (*4*,*5*). Serum samples were tested with a goat antihuman immunoglobulin (Ig) G or a goat antihuman IgM fluorescein isothiocyanate conjugate (Sigma Diagnostics, St. Louis, MO, USA). Patient showed seroconversion with a minimum 8× increase in titers of antibodies against *Rickettsia* spp. between the first samples (collected during the febrile period) and the third blood sample (collected 16 days later) (Table).

DNA was extracted from the skin biopsy specimen by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and tested by a battery of PCRs to amplify fragments of the rickettsial genes citrate synthase (*gltA*) (primers CS-78, CS-323, CS-239, CS-1069), outer membrane protein (*ompB*) (primers 120-M59, 120–807), and *ompA* (primers *Rr*190.70p, *Rr*190.602n), as described (*6*). PCR products were purified and sequenced (*4*). Partial sequences were subjected to BLAST analysis (*7*) to determine similarities to other *Rickettsia* species. Partial *gltA* sequence (1,078 bp) showed 100% similarity to *R. sibirica* (RSU59734), 99.9% to *R. parkeri* (EF102236), and 99.8% to *R. africae* strain S (RSU59735). Partial *ompB* sequence (740 bp)

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DOI: 10.3201/eid1603.091338

Table. *Rickettsia* spp. serologic titers by immunofluorescent antibody assay for a Brazilian patient in the state of São Paulo, Brazil, 2009*

	Titers					
	May 19		May 22		Jun 4	
Antigen	IgM	IgG	IgM	IgG	IgM	IgG
Rickettsia rickettsii	64	64	128	256	512	512
R. parkeri	<64	<64	256	256	1,024	512
R. felis	<64	<64	<64	128	<64	128
R. amblyommii	<64	<64	128	<64	512	512
R. rhipicephali	<64	64	128	256	512	512
R. bellii	64	<64	256	128	512	512

*Ig, immunoglobulin.

showed 99.2% similarity to *R. africae* (AF123706) and *R. parkeri* strain NOD (EU567179), and 98.6% to *R. parkeri* (AF123717) and *R. sibirica* (AF123726). Partial *ompA* sequence (463 bp) showed 99.8% similarity to *R. africae* strain S (RSU43805), 99.6% to *R. africae* (EU622980), 99.1% to *R. sibirica* (AF179365), and 98.3% to *R. parkeri* (RPU43802).

For each rickettsial gene, partial sequences were aligned with the corresponding sequences of other *Rickettsia* species available in GenBank, and rooted phylogenetic trees were built with PAUP 4.0b10 (*8*) by using the maximum likelihood method with an heuristic algorithm and the transition model + the Γ, transversion model + Γ, and the general time reversible + Γ + proportion invariant model for *gltA, ompB,* and *ompA,* respectively, as determined by Model Test (*9*). Tree stability was assessed by bootstrapping >1,000 replicates. In all trees, the sequence from the Brazilian patient, designated as *Rickettsia* sp. Atlantic rainforest, grouped in a cluster composed by different strains of *R. africae, R. parkeri,* and *R. sibirica.* This cluster was supported by high bootstrap value for *ompB* tree, but low for the *ompA* tree (Figure 2). Little divergence was observed between SFG species in the *gltA* tree; clusters were generally supported by low bootstrap values (data not shown). Partial sequences (*gltA, ompB, ompA*) from *Rickettsia* sp. strain Atlantic rainforest generated in this study were deposited into GenBank and assigned nucleotide accession nos. GQ855235–GQ855237, respectively.

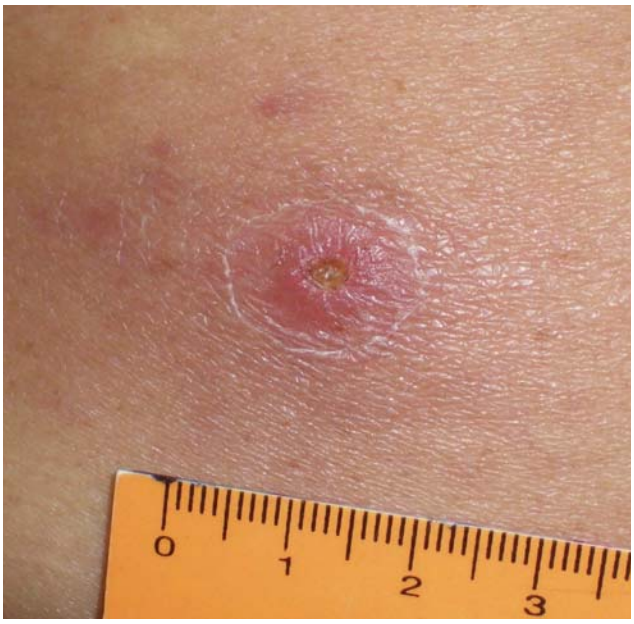

Figure 1. Inoculation eschar on the lumbar region of the back of a patient infected with a *Rickettsia* sp. from the Atlantic rainforest in the state of São Paulo, southeastern Brazil.

Conclusions

We report a clinical case of SFG rickettsiosis acquired in an Atlantic rainforest area of the state of São Paulo, Brazil. Definitive diagnosis is supported by demonstrating a minimum 8× increase in titers between acute-phase and convalescent-phase serum samples, and by identification of rickettsiae in an acute-phase tissue sample (eschar), which was confirmed as a novel SFG strain and designated as *Rickettsia* sp. strain Atlantic rainforest. Genetic analyses indicated that this new strain was similar to *R. africae, R. parkeri,* and *R. sibirica.* The clinical profile of the Brazilian patient was similar to the disease caused by these 3 rickettsial species in the United States (*R. parkeri*) and in the Old World (continents of Asia, Europe, and Africa [*R. africae* or *R. sibirica*]), that is, mild fever, muscle and joint pain, eschar, rash, and no deaths (*10–12*). We did not observe regional lymphadenopathy, a clinical sign usually associated with *R. parkeri, R. africae,* and *R. sibirica* (*10–12*) infection, possibly because the inoculation eschar was on the lumbar region of the back.

It was recently proposed that a new *Rickettsia* species should not show ≥99.9%, 99.2%, and 98.8% similarity for the *gltA*, *ompB*, and *ompA* genes, respectively, with the most homologous validated species (*13*). The strain detected in the Brazilian patient showed similarity values equal to or greater than the above threshold values for ≥2 genes of either *R. africae* or *R. parkeri* or *R. sibirica.* Thus, we cannot identify the species for *Rickettsia* sp. strain Atlantic rainforest. Notably, it has been proposed that closely related species, such as *R. parkeri* and *R. africae*, should be considered strains of 1 species (*12*).

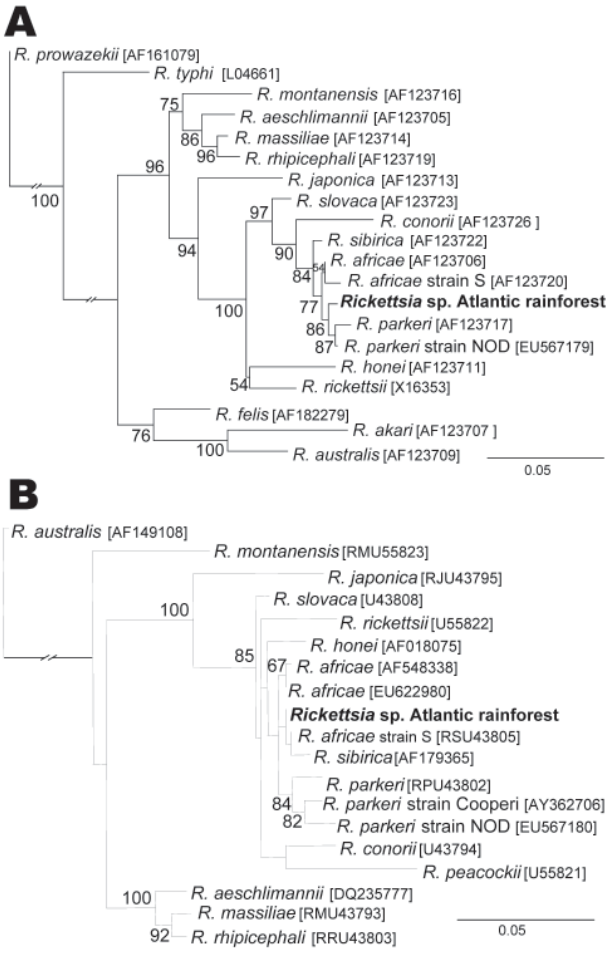

Figure 2. Molecular phylogenetic analysis of *Rickettsia* sp. strain Atlantic rainforest detected in a patient from the State of São Paulo, Brazil. A) A total of 740 unambiguously aligned nucleotide sites of the rickettsial outer membrane protein (*ompB*) gene were subjected to analysis. B) A total of 463 unambiguously aligned nucleotide sites of the rickettsial *ompA* gene were subjected to analysis. Bootstrap values >50% are shown at the nodes. Numbers in brackets are GenBank accession numbers. The strain isolated in this study is indicated in **boldface**. Scale bars indicate nucleotide substitutions per site.

Acknowledgments

We thank Sheila Oliveira de Souza for technical assistance in DNA sequencing.

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico, and the Ministry of Health of Brazil. This work was performed in the Faculty of Medicine, and at the Faculty of Veterinary Medicine of the University of São Paulo, São Paulo, Brazil.

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Climate Warming and Tick-borne Encephalitis, Slovakia

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Increased tick-borne encephalitis (TBE) cases have been reported in central Europe. To investigate temporal trends in the altitude at which TBE cases occur in Slovakia, we analyzed the number of TBE cases during 1961–2004. Since 1980, TBE cases moved from lowlands to submountainous areas, most likely because of rising temperature.

The recent increase in incidence of tick-borne encephalitis (TBE) in central and eastern Europe, especially since 1990, has been attributed to climate warming (*1*–*5*) or various socioeconomic factors (*6*,*7*). Climate warming in Europe during the past decades has been shown to influence the distribution of *Ixodes ricinus* ticks, the main TBE vector, in several European countries (*4*,*5*,*8*). In central Europe, a sharp increase of TBE has been reported (*9*,*10*). Zeman and Beneš showed that global warming affected the geographic and temporal distribution of TBE cases in the Czech Republic (*2*). Similar development of TBE vertical distribution could be expected in neighboring Slovakia. To investigate temporal trends in the altitude at which TBE cases occur (altitude for TBE) in Slovakia and TBE response to climate warming, we analyzed the total number of TBE cases recorded for persons in Slovakia during 1961–2004.

The Study

Since the 1952 outbreak of TBE in Rožňava, Slovakia, all registered cases of TBE have been required to be reported to the National Health Institute. We analyzed 1,786 TBE cases registered in Slovakia by the Regional Institute of Health during 1961–2004.

Location where infection occurred was tracked to the level of cadastral unit. We calculated the average altitude of cadastral units corresponding to the reported TBE cases by using an altitudinal model of the country and ArcGIS 9.2 software (www.ESRI.com). A TBE focus was defined as a location at which TBE infection occurred at least 1 time in a given year. The yearly average altitude for TBE was plotted against time, and temporal trends were identified by linear regression analysis. Frequency distribution of TBE foci in relation to altitude was plotted, and 5-year periods were aggregated. To eliminate locations with single, possibly accidental, cases of the disease, we considered established TBE foci where TBE had occurred in at least 2 of 5 years .The series of yearly mean altitudes of TBE foci was tested against the null hypothesis of random elevation by using the Spearman rank correlation (2-tailed test; null hypothesis = temporal and altitudinal rankings are uncorrelated) and the test for stationarity of Kwiatkowski et al. (*11*) (null hypothesis = time series in question is stationary; i.e., no change over time). A series of yearly mean altitudes for TBE was analyzed for correlations with mean yearly temperature and precipitation derived from 12 meteorologic stations throughout Slovakia. Climate data were kindly provided by the Slovak Hydrometeorological Institute (www.shmu.sk). Statistical tests were performed by using SPSS 14.0 for Windows (Chicago, IL, USA) and Gretl 1.8.5 (*12*).

During 1961–1979, the mean altitude for TBE varied between 180 m and 340 m above sea level. Time series of mean altitudes for TBE showed random elevation, and statistical analysis showed no temporal trend. During this period, no temporal trend in the average annual air temperature was noted. However, during the following period, 1980–2004, the mean altitudes for TBE showed nonrandom variation over time. The gradual increase is shown in Table 1. An analysis of trends (linear least-square fit) showed a mean ± SD annual ascension rate of 5.32 ± 0.63 m; $R^2 = 0.76$, $p<0.001$ (Figure 1). The relationship can be expressed as the following equation: Annual rise in the mean altitude of TBE incidents (meters above sea level) = (222.80 + 5.32) × (year from 1980 inclusive) ± 0.63.

The observed rise in mean altitude for TBE corresponds with a mean ± SD rate of TBE ceiling (uppermost limit) rise of ≈5.4 ± 1.7 m yearly during the past 3 decades in the neighboring Czech Republic (*2*). During the same frame, the mean annual temperature showed a gradual rise (Table 1). An analysis of trends (linear least-square fit) showed an annual increase of 0.067°C ± 0.019°C (R^2 = 0.36, p = 0.002).

Table 1. Nonparametric test and test of stationarity for mean altitude and mean annual air temperature with regard to TBE, Slovakia, 1980–2004*

Data	Test values			
	R_S	p value†	KPSS	p value†
Mean annual air temperature	0.55	0.01	0.56	<0.05
Mean TBE altitude	0.87	<0.001	0.86	<0.01

*TBE, tick-borne encephalitis; Rs, Spearman rank correlation test, a nonparametric test; KPSS, Kwiatkowski-Phillips-Schmidt-Shin test, a test of stationarity (no change with time).
†Probability of adopting the null hypothesis of randomness (Spearman R_S) and stationarity (KPSS).

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DOI: 10.3201/eid1603.081364

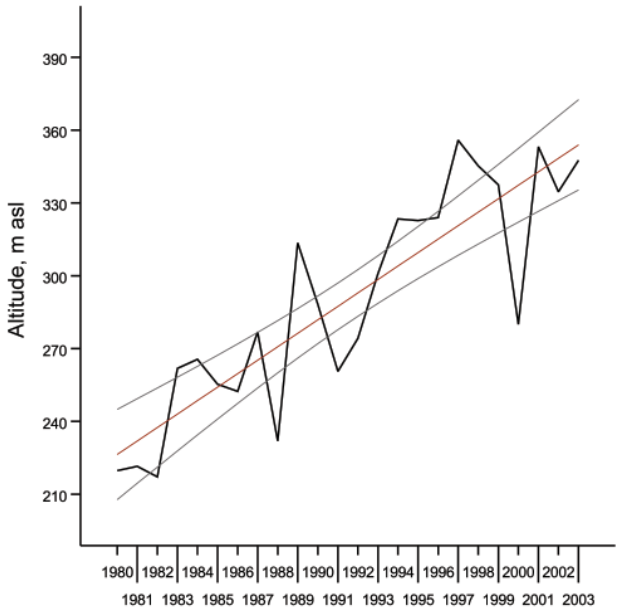

Figure 1. Mean altitude of reported cases of tick-borne encephalitis (TBE), Slovakia, 1980–2004. Black line, mean altitude; red line, linear least-square fit; gray lines, 95% confidence intervals; asl, above sea level.

The mean altitude for TBE in this period was significantly correlated with mean annual air temperature (Table 2). No significant correlation between the mean altitude for TBE and precipitation could be found. The closest correlation was detected between the mean altitude for TBE and mean annual air temperature of the 3 preceding years. This correlation indicates that the mean altitude for TBE positively responds to climate warming, with a lag of several years. A similar phenomenon was described by Zeman and Beneš (*2*). At the beginning of the observed period of change, 1980–1984, 48.6% of TBE foci were found at <200 m (Figure 2), 21.6% were found at >300 m, and the highest with repeated reports of TBE was 550 m. During 2000–2004 only 23.0% of locations with repeated reports of TBE were found at <200 m, 27.8% of all locations were found at >400 m, and 5.6% of all TBE foci were found at >600 m (Figure 2). During this period, the highest location with TBE occurrence repeated for several years was 832 m.

Table 2. Relationship between mean annual air temperature and mean altitude of tick-borne encephalitis cases, Slovakia, 1980–2004*

Temperature lag, y	Correlation coefficient	p value
Mean (1–3)	0.689†	0.000
0	0.30‡	0.032
–1	0.466‡	0.019
–2	0.433‡	0.031
–3	0.438‡	0.028

*Nonparametric testing (Spearman rank correlation test).
†Correlation is significant at p<0.01 (2-tailed).
‡Correlation is significant at p<0.05 (2-tailed).

The total number of lowland TBE foci at <200 m decreased from 36 during 1980–1984 to 29 during 2000–2004.

In contrast, the total number of TBE foci at >400 m was only 2 during 1980–1984 and increased to 35 during 2000–2004. The altitudinal distribution of TBE foci during 1980–1984 differed significantly from that during 2000–2004 (log-likelihood ratio 31.302, df = 7, p<0.001). The number of lowland TBE foci became significantly lower than in the beginning, a finding that corresponds with the predictions of Randolph and Rogers about the gradual disappearance of TBE from the lowlands of central Europe (*7*). The dramatic rise in the number of TBE foci at >400 m between 1980–1984 (2 foci) and 2000–2004 (35 foci) is too great to be explained by only socioeconomic factors, such as particular changes in land use, which could increase the range of habitats suitable for tick survival at higher altitudes.

Conclusions

If the observed trend continues, the number of TBE foci in the mountain areas >500 m will probably increase in future decades. Whether this would affect the total number of TBE cases is a matter for discussion. Higher areas are less densely inhabited by local residents but often visited for leisure activities and recreation. The possibility of TBE emergence should be therefore considered by the management of recreation facilities and tourist resorts in areas with habitats suitable for TBE vectors.

Acknowledgments

We dedicate this work to Milan Labuda, who devoted a great part of his life to the study of TBE in Slovakia and unfortunately passed away before this work was finished. We also thank the

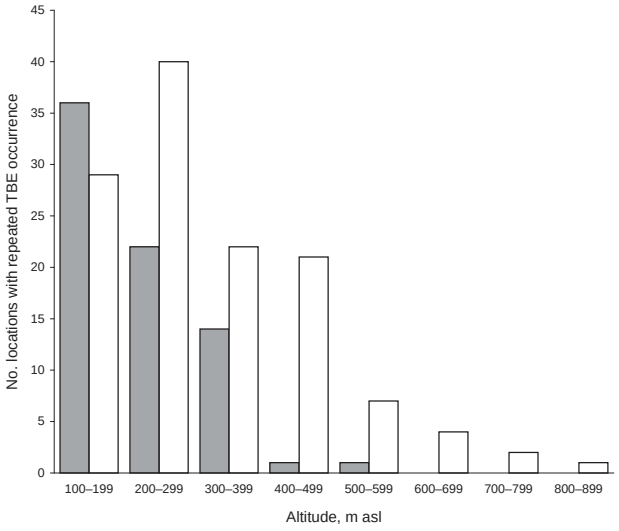

Figure 2. Comparison between altitudinal distribution of tick-borne encephalitis (TBE) foci during 2 time periods, 1980–1984 (gray bars) and 2000–2004 (white bars), Slovakia. asl, above sea level.

Regional Institute of Public Health in Banská Bystrica for support with reports on TBE.

This research work was supported by grant no. 2/6163/26 from VEGA (research grant agency of the Slovak Ministry of Education and Slovak Academy of Sciences).

Dr Lukan is a researcher at the Institute of High Mountain Biology. As a part of his PhD program, he is studying the distribution of *I. ricinus* ticks and tick-borne diseases in Slovakia in response to climate change.

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Terrestrial Rabies and Human Postexposure Prophylaxis, New York, USA

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During 1993–2002, cats accounted for 2.7% of rabid terrestrial animals in New York but for one third of human exposure incidents and treatments. Nonbite exposures and animals of undetermined rabies status accounted for 54% and 56%, respectively, of persons receiving rabies treatments.

Rabies has an almost 100% case-fatality rate and requires considerable resources for control (*1*). In the United States, canine rabies is controlled with vaccination and control of dogs (*2*). Infection occurs primarily from bite wounds. In US cases diagnosed before death, patients died 6–43 days after clinical onset (*3*). Although <10 human cases have been diagnosed annually since 1990 (*2*) in the United States, potential exposure incidents and rabies postexposure prophylaxis (PEP) of humans are not rare. PEP is the treatment regimen for 1 person, with 2–5 vaccine injections and immune globulin, depending on prior vaccination history. PEP is unnecessary if an animal is not rabid at exposure.

A rabies outbreak in raccoons in the mid-Atlantic states in 1977 (*4*) reached New York state, which has many areas with land types favored by raccoons (*5*,*6*), in 1990. In this study, we identified terrestrial rabies trends statewide in New York, with an aim toward prioritizing control. Previous analyses have focused on only part of the state (*7*) or on a shorter time period (*8*).

The Study

In New York, need for PEP is determined by outcome of 10-day confinement (of all domestic animals) or laboratory testing (all species). Healthcare providers report suspected rabies exposures to local health departments, which absorb authorized PEP costs beyond those borne by third-party payers and partial reimbursement by the New York State Department of Health (*9*).

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DOI: 10.3201/eid1603.090298

We analyzed exposure data collected electronically during 1993–2002. Exposures to bats and humans, animals submitted only for surveillance, and data from New York City (not part of the reporting system) were excluded. Rabies was diagnosed by direct fluorescent antibody staining. We analyzed data with SAS version 9.2 (SAS Institute, Cary, NC, USA) using US census data for rates (www.factfinder.census.gov). Because of skewed distributions, we used Spearman rank correlation coefficients for measures of association.

The number of terrestrial animals submitted declined 56% from 10,552 in 1993 to 4,631 in 2002. The number and proportion of rabid animals, which decreased from 2,637 (25.0%) in 1993 to 608 (13.1%) in 2002, were strongly associated with the number of submitted animals (Spearman $r = 0.99$, $p<0.0001$).

For 70.4% of the 13,004 exposure incidents during 1993–2002, an animal was not submitted for testing (Table 1). These incidents accounted for 10,097 (55.6%) of the 18,154 persons receiving PEP. Untestable and positive animals accounted for 2.6% and 23.4% of PEP, respectively. For 3.6% of exposure incidents, PEP began before rabies was ruled out.

Exposure incidents declined 45%, from 1,815 in 1993 to 1,006 in 2001 (Figure 1). PEP decreased from 2,755 (25.3 PEPs/100,000 persons) in 1993 to 1,327 in 2000 (12.1 PEP/100,000 persons). Each year, the number of persons receiving PEP correlated with the number of submitted animals (Spearman $r = 0.94$, $p<0.0001$) and rabid animals (Spearman $r = 0.95$, $p<0.0001$). Although fewer cats (303) than raccoons (8,318) were rabid, cats accounted for the most exposure incidents (4,266 [32.8%]) and PEP (5,777 [31.8%]) (Table 2). Dogs accounted for 3,052 (23.5%) exposure incidents and 3,435 (18.9%) PEP. In New York, dogs and cats accounted for a high proportion of PEP from animals without rabies determination (85.3% and 67.6%, respectively). Raccoons accounted for 3,298 (25.4%) exposure incidents and for 5,210 (28.7%) PEP. From 1993 to 2002, the proportion of PEP attributed to raccoons changed from 48% to 22%; cats, from 21% to 35%; and dogs, from 11% to 22%.

In 43 New York counties with populations <200,000, the PEP rate averaged 33.7/100,000 (range 8.4–81.3/100,000).

Table 1. Terrestrial rabies–associated exposure incidents and rabies PEP use, by animal test result, New York, USA, 1993–2002*

Animal test result	No. (%) incidents	No. (%) PEP uses
Positive	3,047 (23.4)	7,032 (38.7)
Negative	469 (3.6)	551 (3.0)
Untestable	340 (2.6)	474 (2.6)
Not tested	9,148 (70.3)	10,097 (55.6)
Total	13,004 (100.0)	18,154 (100.0)

*Each rabies exposure situation in which ≥1 persons underwent PEP was defined as an incident. Excludes New York, NY. PEP, postexposure prophylaxis.

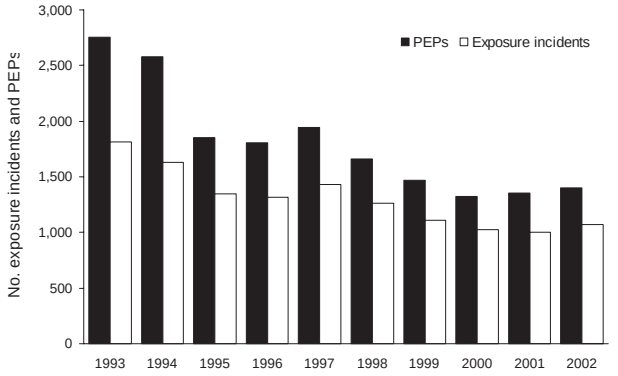

Figure 1. Terrestrial rabies–associated exposure incidents and postexposure prophylaxis (PEP) use, by year, New York (excluding New York City), USA, 1993–2002.

The 14 larger counties (populations >200,000) had significantly lower PEP rates (9.8/100,000, range 0.5–21.8/100,000; p<0.0001) and PEP per exposure incident (p<0.0001) but accounted for 42.6% of PEP.

During 1998–2002 when sex and age of exposed persons were reported, data were missing for 211 of 7,221 PEP reports. Persons who received PEP did not differ by sex (3,625 male, 3,569 female). PEP rates were highest for children 10–14 years of age (Figure 2). For male patients, PEP rates were lower in older age groups; for female patients, rates were highest in the 40–44-year group. Female patients received PEP significantly more often because of cat exposures than did male patients (1,736 vs. 1,053; p<0.0001). Male patients received PEP significantly more often from dog (984 vs. 583; p = 0.0005) and raccoon (767 vs. 595; p = 0.05) exposures than did female patients. For each age group, except the ≥85-year age group, female patients received PEP more often from cat exposures and male patients more often from dog exposures.

The 8,405 bites accounted for 46.3% of PEP. A total of 1,114 (6.1%) of PEP occurrences were associated with scratch exposures and 3,707 (20.4%) with saliva/nervous tissue exposures. For indirect or unknown types of contact, 4,298 (27.2%) PEP occurred. PEP for direct contact significantly exceeded that for indirect or unknown contact for the study period (p<0.0001) and for each year except 1993. Bites accounted for significantly more PEP because of dog and cat exposures (86.4% vs. 63.3%; p<0.0001) than did scratches or saliva/nervous tissue exposures. Raccoon exposures more frequently resulted from saliva/nervous tissue exposure than from bites (22.4% vs. 13.0%; p<0.0001). Most PEP resulting from indirect exposures (64.5%) was from raccoons.

Of 7,221 PEP occurrences during 1998–2002 when local health department authorization was reported, 6,846 (94.8%) were reported as authorized. PEP start date was reported for 6,786 (94.0%). Of 6,264 persons not reported as previously vaccinated, 5,574 (89.0%) received 5 vaccine doses and 5,563 (88.8%) received human rabies immune globulin. Of 522 persons previously vaccinated, 507 (97.1%) received 2 vaccine doses.

PEP completion was not reported (no report received) for 716 (11%) persons; 701 had no prior treatment history. Most (79%) incomplete PEP in New York was associated with animals not captured for rabies determination. Of 119 PEP associated with rabies-negative animals, 108 (91%) were not completed. PEP were not started for 17 (1%) and were not completed for 34 (2%) of the 2,217 PEP associated with rabid animals. Completion rates did not differ by patient sex. Most (697 [97%]) incomplete PEP was from direct contact exposures, primarily bites (87%). A total of 33 (9%) of 376 persons with adverse reactions did not complete treatment. Incomplete PEP was associated more often with exposures to dogs (42%) and cats (42%) than to other species.

The rate in New York was lower than that in Massachusetts when its epizootic was well established in 1995 (*10*),

Table 2. Terrestrial rabies–associated exposure incidents, number of rabid animals, and PEP use, by type of animal, New York, USA, 1993–2002*

Animal	No. exposure incidents	Total no. rabid animals	PEP use		
			Total no. uses	No. related to untested animals	No. related to nonbite incidents†
Wild					
Raccoon	3,298	8,318	5,210	1,488	4,534
Fox	398	390	620	187	318
Skunk	637	1,894	987	302	839
Other	544	152	655	453	328
Domestic					
Dog	3,052	28	3,435	2,930	467
Cat	4,266	303	5,777	3,907	2,119
Other	187	143	668	63	625
Other/unknown	622	7	802	767	519
Total	13,004	11,235	18,154	10,097	9,749

*Each rabies exposure situation in which ≥1 persons underwent PEP was defined as an incident. Excludes New York, NY. PEP, postexposure prophylaxis.
†Scratches, saliva/nervous system tissue exposure, mucous membrane exposure, indirect exposure, or unknown.

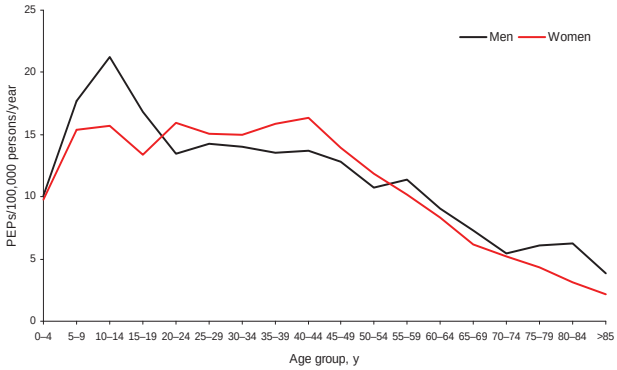

Figure 2. Rate of postexposure prophylaxis (PEP) use per 100,000 persons per year, by sex and 5-year age groups, New York (excluding New York City), 1998–2002.

perhaps because New York requires treating physicians to consult with local public health authorities. Similar to rates in New York, PEP rates in Ontario, Canada, decreased as fox rabies became enzootic and were weakly but significantly associated with animal rabies (*11*). This association may be due to epizootic-related reductions in animal populations, resulting in fewer rabid animals and human contacts. Unlike New York, in Kentucky PEP occurred more frequently after exposures to dogs than cats (*12*). In Kentucky, the proportion of incomplete PEP was the same as in New York (Michael Auslander, pers. comm., 2008). Treatment completion rates for New York and Kentucky were higher than those in a study of 11 US emergency departments (65%) (*13*). In Florida, 22% of PEP were inappropriate according to a state algorithm (*14*); in New York, local health departments report few unauthorized PEP administrations.

Conclusions

In New York, over time and with education, PEP associated with indirect exposures apparently can be reduced. Of most concern is the 55.6% of PEP associated with animals of undetermined rabies status. More efforts are needed to capture exposing animals to rule out both rabies and the need for PEP. Capturing exposing animals should be a major component of animal control efforts that along with vaccination have been successful at reducing rabies risks.

Acknowledgments

We thank the New York State Department of Health Wadsworth Center's Rabies Laboratory and its staff, under the direction of Charles Trimarchi (former director) and Robert Rudd (current director), for collecting the rabies laboratory data. We also thank local health departments for collecting and reporting the exposure and PEP data and the New York State Department of Health Zoonoses Program staff for managing the data.

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Ms Bingman completed this study as her thesis for a master of science degree from the University at Albany School of Public Health. Her research interests include the epidemiology of zoonoses and tuberculosis.

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Increasing Skin Infections and *Staphylococcus aureus* Complications in Children, England, 1997–2006

Sonia Saxena, Paula Thompson, Ruthie Birger, Alex Bottle, Nikos Spyridis, Ian Wong, Alan P. Johnson, Ruth Gilbert, and Mike Sharland, on behalf of improving Children's Antibiotic Prescribing Group

During 1997–2006, general practitioner consultations for skin conditions for children <18 years of age in England increased 19%, from 128.5 to 152.9/1,000 child-years, and antistaphylococcal drug prescription rates increased 64%, from 17.8 to 29.1/1,000 child-years. During the same time period, hospital admissions for *Staphylococcus aureus* infections rose 49% from 53.4 to 79.3/100,000 child-years.

Staphylococcus aureus infection is a leading cause of staphylococcal bacteremia in adults (*1*) and children (*2*) in hospitals in the United Kingdom, and recent reports suggest invasive staphylococci are emerging from the community (*3*). Flucloxacillin is the antimicrobial drug recommended for treating *S. aureus* skin infection in UK primary care centers (*4*). Therefore, its use provides a proxy marker of *S. aureus* skin infection in children. Flucloxacillin prescribing in children has increased over the past 15 years (*5*), despite well-documented reductions in prescribing rates for other commonly prescribed antibacterial drugs during 1995–2000 (*6*), which suggests that *S. aureus* skin infections in the community may be increasing.

We examined the incidence of local complications of *S. aureus* disease in children over a 10-year period using nationally representative data from primary care clinicians in England. Ethics approval for this study was obtained from the Independent Scientific and Ethical Advisory Committee, application no. 2006/ISEAC/012.

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DOI: 10.3201/eid1603.090809

The Study

The MediPlus UK database contains anonymized longitudinal data from >500 UK general practitioners who contribute clinical data on >1 million patients (*7*) that have been used widely for research (*8*). Consultations are coded by using the International Classification of Diseases, Tenth Revision (ICD-10), and antimicrobial drug prescriptions are coded by using the British National Formulary for children, Chapters 5.1.1–5.1.3 (*9*). Using Mediplus UK, we extracted data on all skin conditions (ICD-10 code) and atopic dermatitis (ICD-10 code L20) as an index condition in children <18 years of age who saw general practitioners in England from January 1, 1997, through December 31, 2006. We counted prescriptions for all oral and topical antibacterial drugs prescribed for skin infections, and used all oral preparations containing flucloxacillin prescribed for skin conditions as a proxy measure of unresolved *S. aureus* skin infection. We calculated age–sex adjusted annual consulting and prescribing rates by totaling the number of consultations or prescriptions and dividing by the number of person–years contributed by each child in the registered population for each calendar year. We then directly standardized these rates by using the age–sex distribution for the reference year 2000.

The Hospital Episode Statistics (HES) database has recorded all inpatient hospital activity in National Health Service hospitals across England since 1989 and is used widely to monitor disease trends in England (www.hesonline.nhs.uk) (*10*). The main reason for admission, i.e., primary diagnosis, is recorded by using ICD-10 codes. We used HES data to calculate age–sex adjusted admission rates per 100,000 resident population for children <18 years of age for each calendar year from January 1, 1997, through December 31, 2006, for conditions commonly caused by *S. aureus*, including septic arthritis (ICD-10 codes M00.0 for staphylococcal arthritis and M00.9 for pyogenic arthritis), osteomyelitis (M86), and locally invasive skin infections (L02, cutaneous abscesses and boils; L03, cellulitis). Rates were calculated as the total number of admissions per year divided by the mid-year estimate of the number of children residing in England (using the 2000 population in England as the reference population) (*11*). Confidence intervals (CIs) were generated with a Poisson approximation. We used linear regression to test for linear trends in age–sex adjusted admission rates across the period. We used Stata version 9 software (Stata Corp., College Park, TX, USA) for all statistical analysis.

The Mediplus database contained 2,821,372 child-years of follow-up during 1997–2006. General practitioner consultation rates for all skin conditions in children rose from 128.5 (95% CI 127.2– 129.8) per 1,000 child-years

to 152.9 (95% CI 151.4–154.5) per 1,000 child years (p = 0.011). Atopic eczema consultation rates decreased during this time (Figure 1).

In parallel with the rising number of skin consultations was a 64% increase in prescribing rates for antistaphylococcal drugs (flucloxacillin), from 17.8 (95% CI 17.3–18.3) to 29.1 (95% CI 28.5–29.8) prescriptions per 1,000 child-years (p<0.001) (Figure 2). Prescribing of all other antibacterial drugs for children for any reason decreased from 541.4 (95% CI 538.8–544.1) per 1,000 child-years to 484.3 (95% CI 481.6–487.0) per 1,000 child-years (Table 1). Flucloxacillin was the most commonly prescribed antibacterial drug for all skin conditions (37%). Prescribing rates for other classes of anti-bacterial drugs used for skin infections, notably, combined preparations of amoxicillin and clavulanic acid 2% and fusidic acid (<2%), were stable over the time period (Figure 2).

During 1997–2006, unplanned hospital admission rates for skin, bone, and joint infections in all children increased by 49% from 53.4 (95% CI 52.1–54.7) to 79.3 (95% CI 77.7–80.9) per 100,000 child-years (p<0.001) including cellulitis (67.8% increase; p<0.001), skin abscesses (36.7% increase; p <0.001), and osteomyelitis (46.1% increase; p = 0.004) (Table 2). This trend was consistent across all age groups. Admission rates for septic arthritis increased but the result of the test for trend was not significant (p = 0.128).

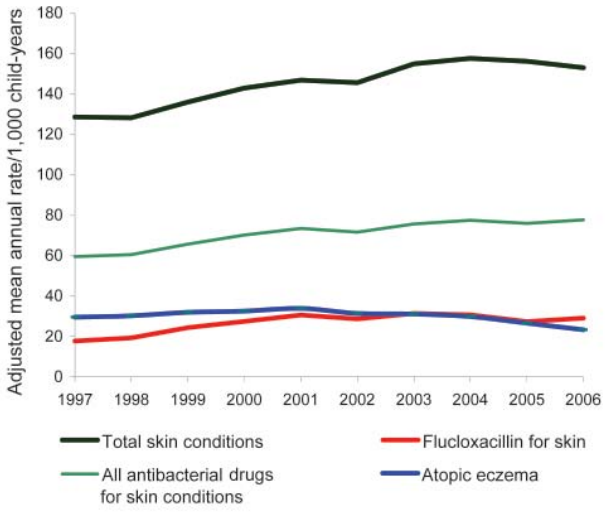

Figure 1. General practitioner consultation and prescribing rates for all skin conditions in children <18 years of age, England, 1997–2006.

Conclusions

The increasing incidence of childhood skin infections and prescribing of the major antistaphylococcal drug flucloxacillin seen in UK primary care, coupled with concurrent increases in childhood hospital admissions for skin bone and joint infections caused by *S. aureus* in hospitals in England, suggests an increase in community-onset *S. aureus* disease in England over the past 10 years. The time frame, a large nationally representative population, use of prospectively collected data, and consistency of patterns make it unlikely our findings arose by chance. Because HES data records primary diagnosis when patients are admitted, most infections will be community-, not hospital-, acquired. Flucloxacillin has remained the treatment of choice for *S. aureus* skin infections in UK primary care for decades (*9*), and its increased use is not explained by treatment drift from other groups of antibacterial drugs used to treat skin infections or by increasing antibacterial drug treatment of atopic eczema colonized with staphylococci. Total unplanned admission rates in children <19 years of age have increased by 13% during 1997–2006 (*12*), but these increases are modest compared with the 49% increase in *S. aureus* complications seen over the same time frame in our study.

Limitations of our study include the use of clinically coded proxy measures for *S. aureus* infections that are subject to recording bias. Because of the lack of microbiologic surveillance, we could not differentiate whether increases in *S. aureus* disease were caused by methicillin-sensitive *S. aureus* or methicillin-resistant *S. aureus*. Antimicrobial drugs for skin infections are available only by prescription in the UK but not exclusively from GPs. Thus, our prescribing data excluded prescriptions issued from other healthcare settings. Our findings that admission rates for osteomyelitis, boils, and cellulitis increased but septic arthritis rates were stable might be because septic arthritis is also caused by pneumococci, β-hemolytic streptococci, and gram negative organisms (*13*).

A growing body of evidence supports our findings of increases in community-onset *S. aureus* disease in children. Hospitalizations for *S. aureus* disease in all age groups are

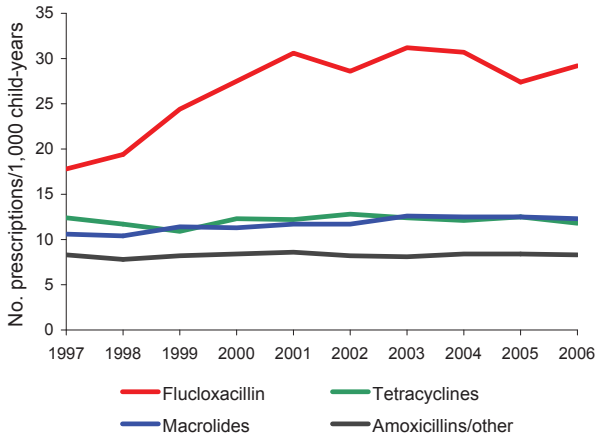

Figure 2. Prescribing rates for antibacterial drugs for children <18 years of age, England, 1997–2006.

Table 1. Age- and sex-adjusted skin condition consultation and antibacterial drug prescribing rates for children <18 years of age, England, 1997–2006 *

Year	Rate/1,000 child-years† (95% CI)				
	GP consultations for skin conditions	Antibacterial drugs for skin conditions	Flucloxacillin for skin conditions	All flucloxacillin	All antibacterial drugs
1997	128.52 (127.21–129.83)	59.53 (58.63–60.43)	17.76 (17.28–18.25)	35.04 (34.36–35.72)	541.42 (538.75–544.09)
1998	128.17 (126.87–129.47)	60.44 (59.54–61.34)	19.33 (18.83–19.84)	37.35 (36.65–38.05)	498.64 (496.09–501.20)
1999	135.91 (134.57–137.24)	65.68 (64.74–66.61)	24.39 (23.83–24.96)	44.03 (43.27–44.79)	438.24 (435.84–440.63)
2000	142.80 (141.44–144.17)	70.17 (69.21–71.13)	27.46 (26.86–28.06)	48.89 (48.09–49.69)	431.80 (429.42–434.18)
2001	146.75 (145.36–148.14)	73.43 (72.45–74.41)	30.65 (30.01–31.28)	52.76 (51.93–53.60)	460.68 (458.21–463.14)
2002	145.56 (144.17–146.94)	71.64 (70.68–72.61)	28.70 (28.09–29.32)	51.39 (50.56–52.21)	443.77 (441.34–446.19)
2003	154.81 (153.37–156.24)	75.69 (74.69–76.68)	31.39 (30.74–32.04)	56.00 (55.13–56.87)	459.94 (457.45–462.43)
2004	157.56 (156.07–159.05)	77.51 (76.47–78.55)	30.75 (30.09–31.42)	56.22 (55.32–57.12)	458.60 (456.04–461.16)
2005	156.05 (154.54–157.57)	75.98 (74.93–77.04)	27.32 (26.68–27.96)	53.58 (52.68–54.47)	489.24 (486.55–491.93)
2006	152.94 (151.44–154.45)	77.62 (76.54–78.69)	29.13 (28.47–29.80)	56.07 (55.15–56.99)	484.30 (481.61–486.98)
p value‡	0.011	0.005	0.001	0.001	0.005

*Data from International Marketing Systems Health. CI, confidence interval; GP, general practitioner.
†Directly age-sex adjusted to 2000 sample population.
‡p value test for linear trend across years.

increasing; in several countries severe skin infections, particularly among children, are rising, caused by strains of *S. aureus* producing the Panton-Valentine leukocidin (*14*,*15*). Although currently no formal surveillance of this strain in the UK is available, referrals of isolates of *S. aureus* positive for Panton-Valentine leukocidin to the national Staphylococcal Reference Unit increased each year from 224 in 2005 to 1,361 2007. What is not known is whether *S. aureus* community-acquired infections in children have added to the recently reported increases of *S. aureus* infection and bacteremias acquired in hospital settings (*4*). Further work is required to monitor *S. aureus* disease and antimicrobial drug resistance and to identify community risk factors for *S. aureus* disease in children.

Acknowledgments

We thank International Marketing Systems Health for providing access to the Mediplus UK data and all the members of the improving Children's Antibiotic Prescribing group.

Table 2 Age- and sex-adjusted hospital admission rates for skin, bone, and joint infections in children <18 years of age, England, 1997–2006*

Year	Rate/100,000 child-years (95% CI)				
	All skin, bone, and joint infections	Cutaneous abscesses and boils	Cellulitis	Osteomyelitis	Septic arthritis
1997	53.38 (52.07–54.70)	25.19 (24.29–26.10)	19.81 (19.01–20.61)	4.81 (4.42–5.21)	3.57 (3.23–3.91)
1998	57.99 (56.62–59.36)	27.37 (26.43–28.32)	22.24 (21.39–23.09)	4.82 (4.43–5.22)	3.55 (3.21–3.89)
1999	61.34 (59.93–62.75)	28.86 (27.89–29.82)	23.63 (22.75–24.50)	5.24 (4.82–5.65)	3.62 (3.28–3.96)
2000	64.95 (63.49–66.41)	28.94 (27.97–29.91)	26.48 (25.55–27.41)	5.70 (5.27–6.14)	3.83 (3.47–4.18)
2001	61.99 (60.56–63.41)	28.02 (27.06–28.98)	25.25 (24.34–26.16)	5.55 (5.12–5.97)	3.17 (2.85–3.50)
2002	63.65 (62.20–65.09)	29.65 (28.66–30.64)	25.01 (24.11–25.92)	5.45 (5.02–5.87)	3.54 (3.19–3.88)
2003	71.15 (69.62–72.68)	32.71 (31.68–33.75)	29.26 (28.27–30.24)	5.39 (4.97–5.81)	3.79 (3.43–4.14)
2004	73.66 (72.11–75.22)	32.98 (31.94–34.02)	30.63 (29.63–31.64)	6.11 (5.66–6.56)	3.94 (3.58–4.30)
2005	76.26 (74.67–77.84)	35.17 (34.10–36.25)	32.03 (31.00–33.05)	5.63 (5.20–6.07)	3.43 (3.09–3.76)
2006	79.28 (77.66–80.89)	34.43 (33.37–35.49)	33.23 (32.18–34.28)	7.04 (6.55–7.52)	4.58 (4.19–4.97)
p value†	<0.001	<0.001	<0.001	0.004	0.128

*Data from Hospital Episode Statistics. Rates directly adjusted to mid-year 2000 resident population for England. International Classification of Diseases, Tenth Revision, codes for primary diagnosis at admission: cutaneous abscesses and boils, L02; cellulitis, L03; osteomyelitis, M86; septic arthritis (staphylococcal arthritis, M00.0, or pyogenic arthritis, M00.9). All of these codes were included in the category "all skin, bone, and joint infections." CI, confidence interval.
†p value for test for linear trend across years.

S.S. has a postdoctoral award from the National Institute for Health Research (NIHR). I.W.'s post was funded by a Department of Health Public Health Career Scientist Award. N.S.'s post is funded by the European Society for Pediatric Infectious Diseases. We are grateful for support from the NIHR Biomedical Research Centre funding scheme.

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Influenza A Pandemic (H1N1) 2009 Virus Infection in Domestic Cat

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Influenza A pandemic (H1N1) 2009 virus continues to rapidly spread worldwide. In 2009, pandemic (H1N1) 2009 infection in a domestic cat from Iowa was diagnosed by a novel PCR assay that distinguishes between Eurasian and North American pandemic (H1N1) 2009 virus matrix genes. Human-to-cat transmission is presumed.

Influenza viruses are typically host specific; aquatic birds are considered the primary reservoir. However, interspecies transmission does occur (*1*–*9*) and occasionally leads to novel host-adapted strains. Interspecies transmission of influenza virus has been a public health concern because of the possibility that, through reassortment, a novel strain with zoonotic potential could emerge. The recent infection of dogs with equine influenza virus (H3N8) (*2*) and of swine with human influenza virus (H1N2) (*4*) are particularly intriguing because the former resulted in influenza becoming endemic in dogs and the latter resulted in a documented reassortment event between human and swine influenza viruses. Such concern has escalated with the recent emergence of the novel quadruple-reassorted influenza virus (H1N1) [pandemic (H1N1) 2009] in humans (*10*). Although infection and transmission of the virus have occurred primarily among humans, occasional transmission from infected persons to susceptible animals (e.g., swine, turkeys, ferrets) has been documented (*11*). The likelihood of pandemic (H1N1) 2009 infection of domestic pets has been considered less likely (www.cdc.gov/h1n1flu/qa.htm, www.avma.org/public_health/influenza/new_virus/default.asp, www.usda.gov/wps/portal/?navid=USDA_H1N1); however, we report a confirmed case of pandemic (H1N1) 2009 virus infection in a domestic cat that had been in contact with persons who had recently experienced influenza-like illness.

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DOI: 10.3201/eid1603.091737

The Case

A 13-year-old, castrated male, domestic cat that lived indoors in a single-cat household was brought to the Iowa State University Lloyd Veterinary Medical Center because of depression, inappetance, and respiratory signs of 4 days' duration. The cat was gregarious and interacted closely with family members in the household. The family members noted that the cat was reluctant to lie in lateral recumbency and instead rested in sternal recumbency with neck extended, which was indicative of dyspnea. The cat's vaccination status was up to date. Before the onset of clinical signs in the cat, 2 of the 3 family members had experienced an undiagnosed influenza-like illness—an upper respiratory tract infection characterized by fever, coughing, and myalgia—that lasted 3 days. Onset of the cat's clinical signs was noted 6 and 4 days after onset of illness for the first and second family members, respectively.

At the time of examination, the cat had bilateral adventitial lung sounds (wheezes), was afebrile, and was clinically dehydrated. Radiographs of the thorax showed a bilateral caudodorsal alveolar pattern (Figure). Cytologic and microbiologic examination of bronchoalveolar lavage (BAL) fluid showed foamy macrophages (65%), nondegenerate neutrophils (25%), and small lymphocytes (10%). Clinicopathologic findings suggested a moderate, predominantly macrophagic, mixed inflammatory process. Standard microbial culture of BAL aliquots yielded no substantial growth of aerobic or anaerobic bacteria. Radiographic and cytologic findings were inconsistent with bacterial or parasitic pneumonia and not supportive of allergic airway disease. A viral cause was considered most likely; however, the cat was given amoxicillin with clavulanate (125 mg orally 2×/day) to reduce the possibility of secondary bacterial pneumonia. Notable findings from laboratory testing (complete blood count, serum biochemistry, urinalysis, and total thyroxine measurement) were moderate leukopenia characterized by a moderate lymphopenia, modest hemoconcentration, and a slightly elevated thyroxine level. Lymphopenia was consistent with acute viral infection.

PCR testing (Feline URD Panel; Idexx Laboratories, Westbrook, ME, USA) of a BAL sample showed negative results for *Chlamydophila felis,* feline calicivirus, feline herpesvirus-1, *Bordetella bronchiseptica,* and *Mycoplasma felis*. Results of feline immunodeficiency virus (antibody) and feline leukopenia virus (antigen) testing (Idexx SNAP FIV/FeLV Combo Test; Idexx Laboratories) were also negative, ruling out the potential that viral-induced immunosuppression was a concurrent factor. For the following reasons we included pandemic (H1N1) 2009 on our list of

differential diagnoses: recent history of respiratory disease in household family members, known widespread community prevalence of pandemic (H1N1) 2009 influenza in humans, paucity of common viral infections causing infectious caudodorsal alveolar pneumonia in adult cats, and documented susceptibility of felids to avian influenza (H5N1) (*12*,*13*). We therefore submitted a BAL sample to the Iowa State University Veterinary Diagnostic Laboratory for molecular screening and typing for influenza A and the pandemic (H1N1) 2009 virus.

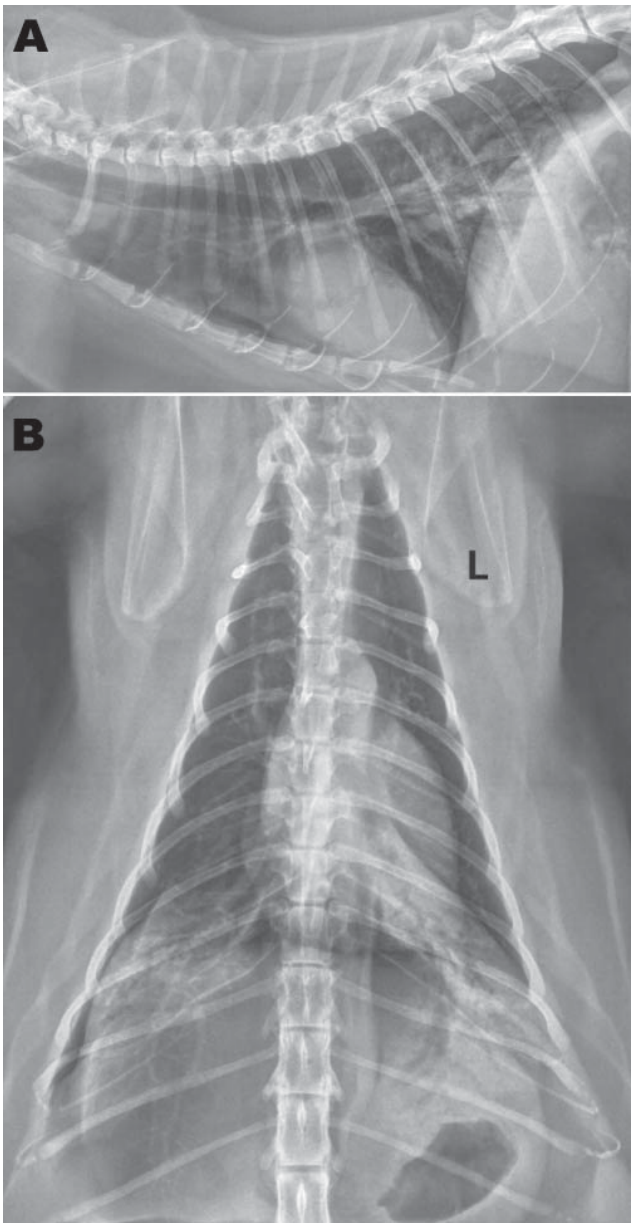

Figure. Radiographs of the thorax of a cat with confirmed influenza A pandemic (H1N1) 2009 virus infection. A) Right lateral view; B) dorsoventral view. Asymmetric soft tissue opacities are evident in the right and left caudal lung lobes. An alveolar pattern, composed of air bronchograms with border-effaced (indistinct) adjacent pulmonary vessels, is most pronounced in the left caudal lobe. A small gas lucency in the pleural space appears in the right caudal and dorsal thoracic cavity. An endotracheal tube is visible at the thoracic inlet on the lateral view in this moderately obese cat. L, left.

RNA was obtained from the BAL fluid by using the MagMAX Viral RNA Isolation Kit (Applied Biosystems, Austin, TX, USA) and a semiautomated magnetic particle processor (Kingfisher 96; Thermo Electron Corp., Woodstock, GA, USA) according to manufacturer's recommendations. Molecular testing used a real-time reverse transcription–PCR (rRT-PCR) influenza A screening assay specific for the nucleoprotein gene. Preliminary differentiation of pandemic (H1N1) 2009 virus from other H1 or H3 types of influenza A was performed by using an in-house rRT-PCR assay that distinguishes between pandemic (H1N1) 2009 [Eurasian matrix (*10*)] and endemic (to North America) swine H1N1 influenza viruses (North American matrix). Sequences of primers and probes are summarized in Table 1. PCRs were conducted by using the AgPath-ID Multiplex One-Step RT-PCR Kit (Ambion/Applied Biosystems) according to manufacturer's recommendations; 10 units of Multiscribe Reverse Transcriptase (Applied Biosystems) were added per reaction. Thermocycling was performed by using the Applied Biosystems 7500 Fast Real-Time PCR System according to manufacturer's recommendations.

PCR testing showed the BAL sample to be positive for influenza A virus (nucleoprotein gene), and the virus was determined to contain the matrix (M) gene of the pandemic (H1N1) 2009 virus strain. A BAL sample was submitted to the US Department of Agriculture National Veterinary Services Laboratories (Ames, IA, USA) for confirmatory testing. rRT-PCR confirmed that the BAL sample was positive for the M gene of influenza A virus and the neuraminidase (N) gene of pandemic (H1N1) 2009 virus. Sequences of primers and probes are summarized in Table 2. A cytolytic virus was isolated by using MDCK cells (*8*) and was designated as A/feline/IA/NVSL026991/2009. PCR testing of the isolate for influenza A virus (M gene) and N1 gene of pandemic (H1N1) 2009 showed positive results. Sequence analyses for hemagglutinin (HA), N, and M genes confirmed that the virus was pandemic (H1N1) 2009 virus (GenBank accession nos. GU332630 (for HA), GU332632 (for NA), and GU332631 (for M). Nucleotide homologies with the first US human pandemic (H1N1) 2009 isolate (A/CA/04/2009) were 99.4%, 99.4%, and 99.8% for the HA, NA, and M genes, respectively.

The cat was discharged from the medical center after diagnostic testing and correction of dehydration. A veterinarian (B.A.S.) visited the home to monitor the cat's clinical status and administer subcutaneous fluids (120–160 mL) until the cat's appetite improved; adventitial lung sounds resolved within 3 days. Reassessment 1 week later showed

Table 1. Oligonucleotide sequences for primers and probes and dye labels used in novel molecular testing for pandemic (H1N1) 2009 virus, Iowa State University Veterinary Diagnostic Laboratory, Ames, Iowa, USA, 2009*

Name	Sequence (5′ → 3′)	Description
Influenza A NP screening assay		
SIVRTF	CGGACGAAAAGGCAACGA	NP forward primer
SIVRTR	CTGCATTGTCTCCGAAGAAATAAG	NP reverse primer
SIVRTP	CCGATCGTGCCYTC	NP probe, MGB FAM
Pandemic influenza M differentiation assay		
M_F	TCAGGCCCCCTCAAAGC	M forward primer
M_R1	CATTCCATGAGAGCCTCAAGATC	M reverse primer 1
M_R1a	CACTCCATGAGAGCCTCAAGATC	M reverse primer 2
M_R1b	CATTCCATGAGTGCCTCAAGATC	M reverse primer 3
M_EUPr	CAGAGACTGGAAAGTGT	EU M MGB, VIC
M_NAPr	CAGAGACTYGAAGAYGT	NA M MGB, FAM

*Primers and MGB probes were obtained from Integrated DNA Technologies (Coralville, IA, USA) and Applied Biosystems Inc. (Foster City, CA, USA), respectively. SIV, swine influenza virus; NP, nucleoprotein; M, matrix.

marked improvement of clinical signs but only modest improvement of the lymphopenia and radiographic findings.

Conclusions

Because the cat was from a single-animal household and remained indoors, he was presumably infected through contact with the family members. Attempts to retrospectively confirm pandemic (H1N1) 2009 infection in the family members have been unsuccessful, but additional testing of archived biologic samples is being conducted. Although more surveillance and studies are needed to determine susceptibility of companion animals to the pandemic (H1N1) 2009 virus, possible reverse zoonotic transmission (humans to animals) remains a concern. Indeed, cases in a domestic dog and other felids have been confirmed (*11*) (www.cdc.gov/h1n1flu/qa.htm, www.avma.org/public_health/influenza/new_virus/default.asp, www.usda.gov/wps/portal/?navid=USDA_H1N1). Implications of pandemic (H1N1) 2009 virus infection in companion animals are 1) apparent human-to-animal transmission; 2) broader host range for the virus; 3) potential endemic establishment of influenza in companion animals; 4) possible transmission of influenza from companion animals to other species, including humans; and 5) the need to reevaluate companion animals as potential reservoirs or intermediate hosts for reassortment of influenza virus. This case emphasizes the need for close monitoring for interspecies transmission of influenza virus and reinforces the need for collaboration among many disciplines, a cornerstone of the One Health Initiative (www.onehealthinitiative.com).

Acknowledgments

We thank the family members in the cat's household for their cooperation and Sarah Abate, Wendy Stensland, and Leslie Bower for technical assistance.

This study was supported by the Iowa State University Office of the Vice President for Research and Economic Development, the Iowa Healthy Livestock Initiative Research Grant, and the Center for Advanced Host Defenses, Immunobiotics and Translational Comparative Medicine.

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Table 2. Oligonucleotide sequences for primers and probes and dye labels used in confirmatory molecular testing for pandemic (H1N1) 2009 virus, National Veterinary Services Laboratories, Ames, Iowa, USA, 2009*

Name	Sequence (5′ → 3′)	Description
Influenza A M screening assay		
M+25	AGATGAGTCTTCTAACCGAGGTCG	AI M forward primer
M-124	TGCAAAAACATCTTCAAGTCTCTG	AI M reverse primer
M-124siv	TGCAAAGACACTTTCCAGTCTCTG	H1N1 M reverse primer
M+64	TCAGGCCCCCTCAAAGCCGA	M probe, BHQ, FAM
Pandemic influenza N1 differentiation assay		
N1 220F	CAACACCAACTTTGCTGC	N1 forward primer
N1 330R	GGAACCGATTCTTACACTGTTGTC	N1 reverse primer
N1 232	CAGTCAGTGGTTTCCGTGAAATTAGC	N1 BHQ, FAM

*Primers and probes were obtained from Integrated DNA Technologies (Coralville, IA, USA) and Biosearch Technologies, Inc. (Novato, CA, USA), respectively. M, matrix; AI, avian influenza; N, neuraminidase.

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School Closure and Mitigation of Pandemic (H1N1) 2009, Hong Kong

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In Hong Kong, kindergartens and primary schools were closed when local transmission of pandemic (H1N1) 2009 was identified. Secondary schools closed for summer vacation shortly afterwards. By fitting a model of reporting and transmission to case data, we estimated that transmission was reduced ≈25% when secondary schools closed.

The emergence and subsequent global spread of pandemic (H1N1) 2009 presents several challenges to health policy makers. Although some countries have substantial antiviral drug stockpiles available for treatment and chemoprophylaxis and vaccines became available toward the end of 2009, nonpharmaceutical interventions remain the primary resource available to most populations to mitigate the impact of pandemic (H1N1) 2009 (*1*). One such nonpharmaceutical intervention is school closure, either reactively following outbreaks or proactively at district or regional levels (*2*,*3*). A recent review has highlighted the lack of consensus over the potential benefits of school closures and the potential economic and social costs (*4*). Although the current pandemic (H1N1) 2009 virus is of moderate severity, data from 2009 provide an ideal opportunity to estimate the effectiveness of interventions against pandemic influenza.

In Hong Kong Special Administrative Region, People's Republic of China, there was a considerable delay between the first reported imported case on May 1, 2009, and the first reported local case (i.e., not otherwise epidemiologically linked with outside travel, contact with an imported case-patient, or contact with an infected person who had contact with an imported case-patient) was laboratory-confirmed and reported to the government on June 10. During the initial stages of the epidemic, the local government operated under containment phase protocols, in which all confirmed cases were isolated in hospital and their contacts were traced, quarantined in hotels, hospitals, and holiday camps, and provided with antiviral drug prophylaxis. When the first nonimported case was confirmed, the government entered the mitigation phase and announced immediate closure of all primary schools, kindergartens, childcare centers and special schools, initially for 14 days. Closures were subsequently continued until the summer vacation began July 10. Secondary schools generally remained open, while those with ≥1 confirmed case were immediately closed for 14 days. Some containment-phase policies, including isolation of cases and prophylaxis of contacts, were maintained until June 27. During our study period, patients seeking treatment for suspected influenza at designated fever clinics and public hospital emergency departments were routinely tested, and pandemic (H1N1) 2009 virus infection was a reportable infectious disease.

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DOI: 10.3201/eid1603.091216

The Study

We analyzed epidemiologic data on laboratory-confirmed pandemic (H1N1) 2009 infections collected by the Hong Kong Hospital Authority and Centre for Health Protection (the e-flu database). The epidemic curve of laboratory-confirmed pandemic (H1N1) 2009 cases showed a biphasic pattern, with a small initial peak in reported cases at the end of June followed by a nadir at the beginning of July and rising incidence after that (Figure, panel A).

We specified an age-structured susceptible-infectious-recovered transmission model to explain the early pandemic (H1N1) 2009 dynamics in Hong Kong (online Technical Appendix, www.cdc.gov/EID/content/16/3/538-Techapp.pdf). We estimated change points in the proportion of symptomatic infections identified and age-specific rates of seeding of infectious cases from overseas. A simple 3-period model for changes in reporting rates provided a parsimonious fit to the data (Figure, panel B). Reporting rates were defined relative to the initial reporting rate. The comparison between the observed and estimated incidence is shown in the Figure, panel C.

We estimated that the relative rate of reporting declined to ≈5.2% of its initial value from June 29 onward (Table). Persons <19 years of age were estimated to be 2.6× more susceptible than the rest of the population. The estimated effective reproductive number was 1.7 before educational institutions for children <13 years of age were closed on June 11, 1.5 between June 11 and July 10 when summer vacation began, and 1.1 for the rest of the summer. The drop in reproductive number was driven by an estimated 70% reduction in intra–age-group transmission concurrent with school closures. The fitted model implies that ≈182,000 persons (2.5%

of the population) had experienced illness associated with pandemic (H1N1) 2009 infection by August 27.

Figure, panel D shows that in the period from the first confirmed local case to the start of summer vacation on July 10, there were a substantial number of cases among older children (whose schools remained open) but few among younger children (whose schools were closed during this period). Only 10% of Hong Kong residents are young children ≤12 years of age, 8% are older children 13–18 years of age, and 82% are adults.

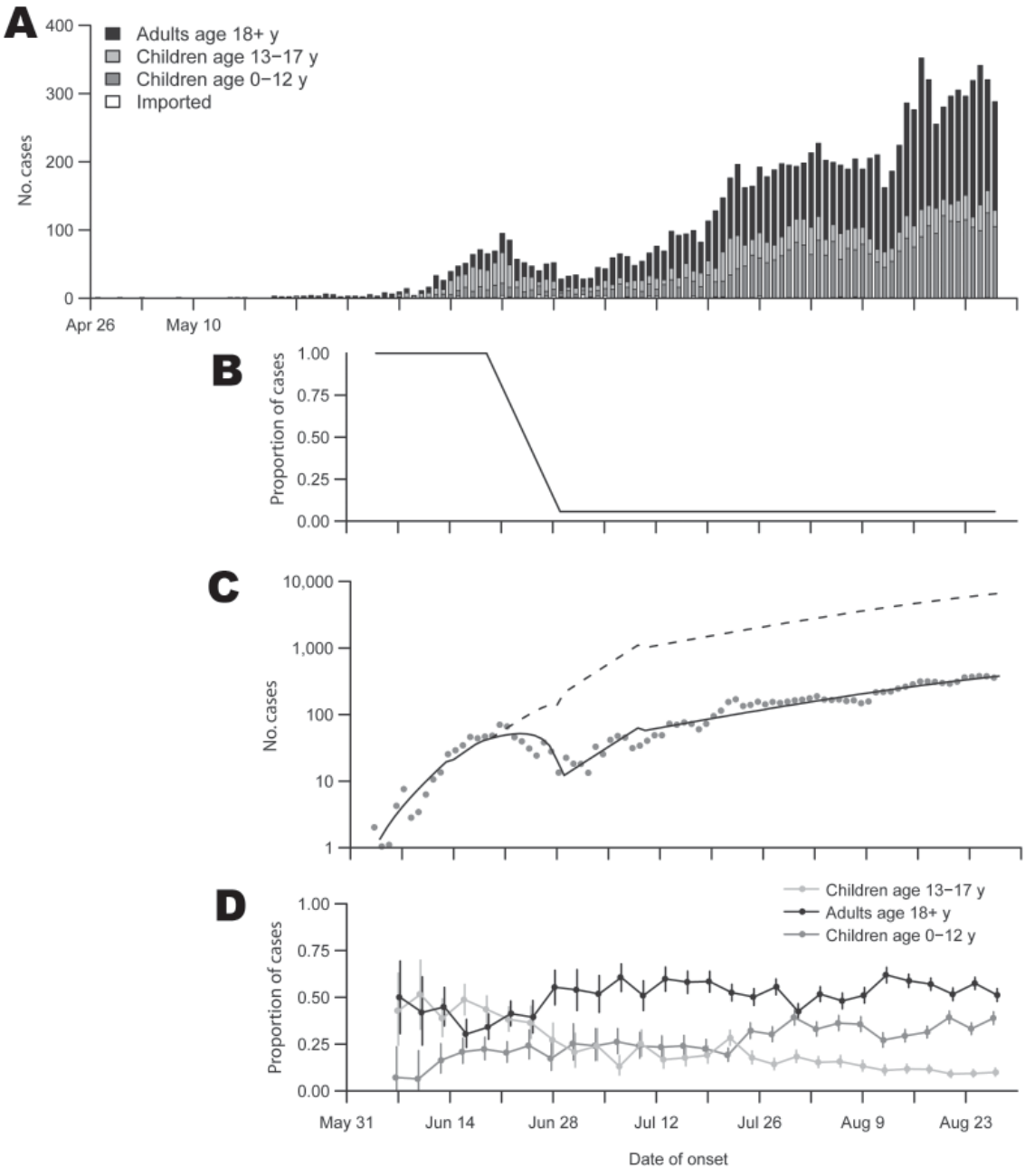

Figure. Epidemiologic characteristics of pandemic (H1N1) 2009 in Hong Kong Special Administrative Region, People's Republic of China, during May through August 2009. A) Time series of laboratory-confirmed pandemic (H1N1) 2009 cases classified as imported or nonimported (by age group) by date of illness onset. B) Estimates of the proportion of cases with illness onset on each day that would subsequently be identified and laboratory confirmed (reporting rates). C) Time series of nonimported pandemic (H1N1) 2009 cases by date of illness onset and the estimates of the underlying true epidemic curve (dashed line) and the fitted observed epidemic curve allowing for changes in reporting rates (solid line). Dots indicate cases reported on a given day. Number of cases plotted logarithmically. D) Distribution of ages of laboratory-confirmed pandemic (H1N1) 2009 cases over time plotted as 3-day rolling averages. Error bars indicate 95% confidence intervals.

Table. Summary statistics of posterior distributions obtained by using Markov Chain Monte Carlo in modeling the effects of school closures on mitigating a pandemic (H1N1) 2009 outbreak, Hong Kong, 2009*

Parameter†	Posterior mean (SD)	95% CI
M_i, daily number of effective seeds in age class i, I = 1,2,3	<13 y: 0.1 (0.1)	0–0.04
	13–19 y: 0.4 (0.1)	0.2–0.6
	>19 y: 0.2 (0.2)	0–0.6
Basic reproductive number	Before Jun 11: 1.71 (0.04)	1.63–1.78
Relative susceptibility of persons <20 y of age	2.64 (0.08)	2.48–2.78
Percentage reduction in intra-age-group transmission given by school closures	70% (3%)	64%–75%
t_1, the date at which reporting rates began to decline	Jun 18 (1.2 d)	Jun 17–Jun 21
t_2, the date at which reporting rates stopped declining	Jun 29 (0.3 d)	Jun 29–Jun 30
r_2, the reporting rate after t_2	5.2% (1.1%)	3.5%–7.7%

*CI, confidence interval.
†Model assumes a linear decline in reporting rates from 100% to r_2 between times t_1 and t_2.

Conclusions

In Hong Kong, kindergartens and primary schools were closed when local transmission of pandemic influenza was identified. By using a parsimonious transmission model to interpret age-specific reporting data, we concluded that the subsequent closure of secondary schools for the summer vacation was associated with substantially lower transmission across age groups. We estimated that reporting of cases declined to 5.2% of its initial rate through the second half of June; this is plausible given the gradual change from containment phase to mitigation phase over that period.

It is challenging to infer the precise impact of school closures in Hong Kong, given that they were implemented immediately and sustained until summer vacation and so we have little data on local transmissibility in the absence of school closures. In previous pandemics attack rates have generally been highest in younger children (*4*,*5*), and this has been noted for pandemic (H1N1) 2009 in Mexico (*6*) and Chicago (*7*). This observation, in combination with our finding that children <12 years of age were relatively unaffected in Hong Kong during the school closure period (Figure, panel D), intuitively implies that closures were effective in preventing infections in this age group. Furthermore, assuming that children are responsible for up to half of all community transmission (*8*), it is likely that protection of younger children had substantial indirect benefits. Previous studies have suggested that sustained school closures during a pandemic could reduce peak attack rates and prevent 13%–17% of total cases in France (*8*) or ≤20% of total cases in the United Kingdom (*3*). Our finding that the reproductive number declined from 1.5 during the kindergarten and primary school closures to 1.1 during summer vacation suggests that a much more substantial drop in attack rates would result from sustained school closures.

By including a model of reporting, we have also been able to estimate case numbers. We estimated a cumulative illness attack rate of ≈182,000 cases (2.5% of the population) by August 27. Between June 29 and August 27, a total of 1,522/9,846 confirmed pandemic (H1N1) 2009 case-patients were hospitalized for medical reasons, among whom 13 died. These numbers are more consistent with a substantially lower case-fatality ratio than suggested by initial estimates of the severity of the pandemic (H1N1) 2009 strain (*9*,*10*). These estimates are dependent on the initial rate of reporting being close to 100%.

We assumed that transmission varied by age and time. If reporting rates varied in a way not accounted for by our model, this would affect the accuracy of our estimates of growth rate and cumulative attack rates. Although we attributed changes in transmissibility between June and August to school closures and summer vacations, it is possible that other secular changes or external factors such as seasonality also contributed. However, it is unlikely that seasonal factors would have reduced transmission of influenza at this time of year, on the basis of symptomatic and laboratory confirmed incidence of influenza from previous years (*11*). Reference data on age-specific population attack rates from serologic surveys or population-based surveillance systems would enable us to calibrate our estimates of reporting rates and growth rates and provide external validation of our model estimates.

Acknowledgments

We acknowledge the Hospital Authority Strategy and Planning Division, Hospital Authority Information Technology Division, and the Centre for Health Protection for the collation of the e-flu database. We thank Max Lau for assistance in drawing the Figure. We thank Kwok Kin On and Danny Yao for helpful discussions.

This research received financial support from the Research Fund for the Control of Infectious Disease, Food and Health Bureau, Government of the Hong Kong Special Administrative Region (grant no. HK-09-04-01); the Harvard Center for Communicable Disease Dynamics from the US National Institutes of Health Models of Infectious Disease Agent Study program (grant no. 1 U54 GM088558); the Area of Excellence Scheme of the Hong Kong University Grants Committee (grant no. AoE/M-12/06); from Fogarty International Center (grant no. 3R01TW008246-01S1); and the Research and Policy for Infectious Disease Dynam-

ics program from Fogarty International Center and the Science & Technology Directorate, Department of Homeland Security.

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Global Origin of *Mycobacterium tuberculosis* in the Midlands, UK

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DNA fingerprinting data for 4,207 *Mycobacterium tuberculosis* isolates were combined with data from a computer program (Origins). Largest population groups were from England (n = 1,031) and India (n = 912), and most prevalent strains were the Euro-American (45%) and East African–Indian (34%) lineages. Combining geographic and molecular data can enhance cluster investigation.

Knowledge and understanding of transmission dynamics of *Mycobacterium tuberculosis* have been improved by development of rapid molecular techniques that are being more extensively applied (*1*–*3*). Globally, application of molecular techniques has identified major *M. tuberculosis* lineages associated with geographic origin (*4*–*7*). Previous studies on transmission dynamics of *M. tuberculosis* have usually analyzed patient-declared population groups to identify associations (*1*,*7*).

We describe a novel software (Origins; Experian, Nottingham, UK) that assigns cultural, ethnic, and linguistic (CEL) groups on the basis of given and family names. Records from 12 countries containing 1,600,000 family and 600,000 given names were analyzed to construct >200 origin types based on CEL factors associated with given and family names. This approach is applicable worldwide and is more accurate and has better coverage than other software (*8*). The first use of Origins in healthcare was identification of how a European CEL group came to emergency departments in the United Kingdom (*9*).

The aim of this study was to combine mycobacterial fingerprinting data and patient origin as assigned by Origins to relate the occurrence of major global *M. tuberculosis* lineages in populations originating from around the world. Combining data obtained from universal typing and associated cultural and social links identified by Origins provides the potential for a deeper understanding of the causes for distribution of prevalent strains in specific population groups.

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DOI: 10.3201/eid1603.090813

The Study

Nonduplicate initial *M. tuberculosis* complex isolates (n = 4,207) were referred from the Midlands region of the United Kingdom (population 9.5 million) to our center during January 2004–December 2007. These isolates were incubated, identified, and analyzed by mycobacterial interspersed repetitive units containing variable numbers of tandem repeats (MIRU-VNTR) typing (*10*). MIRU-VNTR typing analyzes the number of repetitive DNA sequences at multiple independent genetic loci. These data were compared with those in an online database (MIRU-VNTRplus), which was developed by Allix-Beguec et al. (*11*). This database was used to assign *M. tuberculosis* strains to 1 of 6 lineages: East African–Indian, East Asian, Euro-American, Indo-Oceanic, West African-1, or West African-2.

The given and family names of 4,207 patients were entered into Origins to obtain a CEL group for each, which was then assigned a continent on the basis of the United Nations Standard Country and Area Codes Classification Scheme (*12*). Origins can assign a CEL group when the given and family names are present in a dataset.

Within the study population are predominant CEL groups that originate from each continent: 1,031 (25%) from England in Europe, 912 (22%) from India in Asia, and 130 (3%) from Somalia in Africa (Table 1). The 18 isolates from the Americas represented 3 CEL groups. Origins as-

Table 1. *Mycobacterium tuberculosis* isolates from CEL groups, the Midlands, UK*

Continent and CEL group	No. (%) isolates
Africa	263 (6)
Somalia	130 (3)
Other, n = 18 groups	133 (3)
North and South America	
CEL group, n = 3 groups	18 (0)
Asia	2,421 (58)
India	912 (22)
Pakistan	777 (18)
Pakistan–Kashmir	212 (5)
Bangladesh	199 (5)
Northern India	95 (2)
Other, n = 22 groups	226 (5)
Europe	1,473 (35)
England	1,031 (25)
Ireland	123 (3)
Scotland	99 (2)
Wales	98 (2)
Other, n = 23 groups	122 (3)
Unclassified	32 (0)
Total	4,207 (100)

*CEL, cultural, ethnic, and linguistic. CEL groups representing <1% (42 isolates) of the total are not shown.

signed 4,175 (99%) of 4,207 patients to 77 CEL groups; 32 patients were unclassified.

Using the 15 MIRU-VNTR loci, we matched 4,117 (98%) of 4,207 typed strains to strains in the MIRU-VNTRplus database. The 90 strains that did not match with 1 of the 6 major global lineages were *M. bovis* (24 strains) or could not be definitively assigned (66 strains) to 1 of the 6 global lineages. Continental and regional origins of patients as assigned by Origins and global lineage were then combined to identify the distribution of global *M. tuberculosis* lineages within each population (Table 2).

The Euro-American lineage was the most prevalent lineage in our study. It contained 1,894 (45%) strains and was present in each continental human population group. The Euro-American strain was the most prevalent lineage in patients originating from Africa (125), the Americas (11), and Europe (1,072) and was the second most prevalent lineage in patients originating from Asia (663). The most prevalent *M. tuberculosis* lineage in patients originating from Asia was the East African–Indian lineage (1,150).

Combining geographic data assigned by Origins and DNA fingerprinting data could affect public health efforts to control tuberculosis because this approach can identify strains in CEL groups in which specific global *M. tuberculosis* lineages are not present. The MIRU-VNTR profile 424352332515333 (East Asian lineage) was identified in 23 patients from the Midlands. Of these 23 patients, 20 resided within a 5-mile radius of each other. Within this geographically restricted cluster, 12 (60%) of these patients were assigned to the Europe CEL group and 8 patients to part of the Asia CEL group. The first strain was identified in 2004, and subsequent strains were identified in each year of this study.

The MIRU-VNTR profile 422352542517333 was identified in 102 patients during 2004–2007. This profile was matched with the East African–Indian lineage; 98 (96%) patients originated from Asia and 4 (4%) from Europe. This strain was identified in various locations in the Midlands within an ≈40-mile radius that included all patients.

Conclusions

We studied >4,000 *M. tuberculosis* isolates typed in the United Kingdom. Our study demonstrated that the combination of molecular and population group data provided by novel software can provide information about the molecular epidemiology of *M. tuberculosis*.

Table 2. Distribution of *Mycobacterium tuberculosis* isolates according to lineage and continent of patient origin on the basis of CEL group, the Midlands, UK*

Continent and region	No. (%) isolates in each *M. tuberculosis* lineage						Total no. (%) isolates
	East African–Indian	East Asian	Euro-American	Indo-Oceanic	West African-1	West African-2	
Africa							
Eastern	56 (4)	9 (4)	62 (3)	39 (7)	0	0	166 (4)
Central	0	0	4 (0)	1 (0)	0	0	5 (0)
Northern	2 (0)	0	3 (0)	1 (0)	0	0	6 (0)
Southern	2 (0)	1 (0)	29 (2)	3 (1)	0	0	35 (1)
Western	5 (0)	3 (1)	21 (1)	5 (1)	4 (24)	1 (14)	39 (1)
Unknown	0	1 (0)	6 (0)	1 (0)	0	0	8 (0)
Region total	65 (5)	14 (6)	125 (7)	50 (9)	4 (24)	1 (14)	259 (6)
Americas							
Caribbean region	2 (0)	0	5 (0)	0	0	0	7 (0)
North America	2 (0)	0	4 (0)	1	0	0	7 (0)
South America	2 (0)	0	2 (0)	0	0	0	4 (0)
Region total	6 (0)	0	11 (1)	1 (0)	0	0	18 (0)
Asia							
Eastern	4 (0)	25 (11)	10 (1)	5 (1)	0	0	44 (1)
Southeastern	1 (0)	2 (1)	3 (0)	5 (1)	0	0	11 (0)
Southern	1,117 (79)	100 (46)	614 (32)	403 (72)	4 (24)	2 (29)	2,240 (55)
Western	5 (0)	0	13 (1)	1 (0)	0	0	19 (0)
Unknown	23 (2)	1 (0)	23 (1)	5 (1)	0	0	52 (1)
Region total	1,150 (81)	128 (59)	663 (35)	419 (75)	4 (24)	2 (29)	2,366 (58)
Europe							
Eastern	1 (0)	1 (0)	17 (1)	2 (0)	0	0	21 (1)
Northern	186 (13)	69 (32)	994 (52)	74 (13)	7 (41)	4 (57)	1,334 (33)
Southern	9 (1)	6 (3)	40 (2)	6 (1)	1 (6)	0	62 (2)
Western	0	0	14 (1)	0	1 (6)	0	15 (0)
Unknown	1 (0)	0	7 (0)	2 (0)	0	0	10 (0)
Region total	197 (14)	76 (35)	1,072 (57)	84 (15)	9 (53)	4 (57)	1,442 (35)
Unclassified	7 (0)	0	23 (1)	2 (0)	0	0	32 (1)
Total	1,425 (100)	218 (100)	1,894 (100)	556 (100)	17 (100)	7 (100)	4,117 (100)

*CEL, cultural, ethnic, and linguistic. Unknown indicates that the continent was identified but without a specific region. The United Kingdom (Great Britain and Northern Ireland) is located in northern Europe and India, Pakistan, and Bangladesh are located in southern Asia. A total of 90 (2%) of 4,207 strains were not assigned to 1 of the 6 major lineages.

The 2 example MIRU-VNTR profiles show that molecular and social data identified an East Asian strain in an unsuspected CEL group (Europe) and limited transmission of an East African–Indian strain between CEL groups. Geographic restriction of the 424352332515333 East Asian strain in the European CEL group identified possible recent transmission within this population group. The 422352542517333 East African–Indian strain infected a large number of patients (102) and showed wide geographic spread with limited transmission into the European CEL group (4/102 patients). This finding indicates that this strain is widely distributed in southern Asia and has not been transmitted between CEL groups. Its wide distribution in the United Kingdom reflects areas of residence for this CEL group.

Data from our study support previous findings and extend the dataset for Europe. Our results also include a large number of strains from southern Asia, which were underrepresented in other studies (*7*,*13*).

Origins identified CEL groups within a country (e.g., Kashmir in Pakistan or northern India) and divided Great Britain and Ireland into 4 CEL groups (Table 1). This enhanced differentiation could be useful in future population-based studies because migration patterns may be localized to specific areas within countries and common social networks could be identified. CEL groups can be assigned to any dataset in which the patient's name is known. Traditional epidemiologic identification of ethnic groups requires a questionnaire, but if patient names are not in a dataset, then CEL groups cannot be assigned. Origins showed some discrepancies because the black Caribbean CEL group usually has British names and will be assigned as a British CEL group (*8*). However, the utility of Origins is maximized when it is applied to diverse populations.

Many countries now routinely type *M. tuberculosis* isolates by using MIRU-VNTR typing. This analysis identifies clusters of strain types across place and time. By using Origin software for identification of CEL groups, public health officials can identify and investigate possible cultural links for transmission of *M. tuberculosis*.

Acknowledgments

We thank all referring microbiology laboratories in the Midlands for providing specimens and isolates for MIRU-VNTR typing and all staff involved in culture and identification of *M. tuberculosis*. Origins software was originally developed and continues to be maintained and improved by R.W.

This study was supported in part by a grant from the UK Department of Health. Continued development of Origins software is supported by an agreement between R.W. and Experian, the UK and European distributor of the commercial version of this software.

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Quinine-Resistant Malaria in Traveler Returning from Senegal, 2007

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We describe clinical and parasitologic features of in vivo and in vitro *Plasmodium falciparum* resistance to quinine in a nonimmune traveler who returned to France from Senegal in 2007 with severe imported malaria. Clinical quinine failure was associated with a 50% inhibitory concentration of 829 nmol/L. Increased vigilance is required during treatment follow-up.

Resistance of *Plasmodium falciparum* to antimalarial drugs is one the most worrisome problems in tropical medicine. Quinine remains the first-line antimalarial option for treatment of patients with complicated malaria in Europe and Africa. However, emergence of quinine resistance has been sparsely documented (*1*). Maximizing the efficacy and longevity of quinine as a drug to control malaria will critically depend on pursuing intensive research into identifying in vitro markers and implementing active in vitro and in vivo surveillance programs such as those supported by the World Antimalarial Resistance Network. Such molecular markers are needed to monitor temporal trends in parasite susceptibility (*2*). We report quinine-resistant *P. falciparum* malaria in a patient who returned to France from Senegal.

The Patient

A 17-year-old white man from France spent ≈2 months (April and most of May) in 2007 in Dielmo, Senegal, where malaria is highly endemic and shows intense perennial transmission (*3*). He did not use antimalarial prophylaxis or protection against mosquitoes. After returning to France, he was admitted to the Bordeaux University Hospital Center on May 27, 2007 (day 0). The patient had *P. falciparum* parasitemia level of 7% and a 2-day history of fever, myalgia, vomiting, and rapid deterioration of consciousness into an arousable coma. A diagnosis of severe malaria with cerebral involvement was confirmed.

Intravenous quinine formiate (loading dose 17 mg/kg) was administered, followed by a maintenance dose (8.3 mg/kg 3×/day for 7 days). The patient was afebrile on day 3, and his thin and thick blood films became negative for *P. falciparum* on day 6. He was discharged from the hospital on day 7. However, on day 26, he relapsed and had fever and vomiting. He was hospitalized again on day 27 with a core temperature of 40°7C, deterioration of consciousness, and a *P. falciparum* parasitemia level of 4%. He received the same regimen of quinine formiate plus intravenous clindamycin (10 mg 3×/day) for 7 days. His serum quinine level (free and bound drug assayed by high performance liquid chromatography) taken immediately before the fourth drug dose was low (7 mg/L). The patient was then given quinine (10 mg/kg 3×/day from day 30 through day 34). Serum quinine levels then increased and fever cleared within 72 hours. However, a blood smear was positive on day 34.

Because of the treatment failure with quinine and clindamycin, the patient was treated with oral co-artemether (20 mg artemether and 120 mg lumefantrine, given as 4 tablets, followed by 4 tablets after 8 hours, and 4 tablets 2×/day for 2 days; total = 24 tablets). Parasitic clearance was observed within 48 hours. Blood smears and results of a PCR for *P. falciparum* were negative from day 36 through day 62. No further recrudescence occurred over the next 12 months.

The isotopic microdrug susceptibility tests used have been described (*4*). The chloroquine-susceptible 3D7 *P. falciparum* clone (Africa) and the chloroquine-resistant W2 clone (Indochina), after 2 rounds of sorbitol synchronization, were used as controls. The 50% inhibitory concentration (IC_{50}) values for 12 antimalarial drugs for the study isolate and these 2 controls are shown in the Table. The strain isolated on day 27 showed reduced susceptibility to quinine (IC_{50} 829 nmol/L, threshold 800 nmol/L) and chloroquine (472 nmol/L, threshold 100 nmol/L). The IC_{50} for clindamycin was 39 μmol/L (the in vitro resistance cutoff value was not determined). The isolate was susceptible to all other antimalarial drugs tested. Phenotypes and genotypes were assessed only for parasites obtained on day 27.

We concurrently screened blood samples for resistance-associated point mutations. A sequence containing the ms4760 microsatellite was amplified as described (*5*). The observed ms4760–18 profile was composed of 2 DNNND repeats and 2 DDDNHNDNHNN repeats. Genotyping of the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) gene, which encodes a transport protein

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DOI: 10.3201/eid1603.091669

Table. In vitro susceptibility of 3 *Plasmodium falciparum* isolates to 12 antimalarial drugs, France*

Drug	50% Inhibitory concentration			
	Study isolate	3D7	W2	Cutoff value
Quinine	829 nmol/L	157 nmol/L	574 nmol/L	>800 nmol/L
Chloroquine	472 nmol/L	21 nmol/L	392 nmol/L	>100 nmol/L
Mefloquine	10.4 nmol/L	49.3 nmol/L	39.3 nmol/L	>30 nmol/L
Lumefantrine	19 nmol/L	29 nmol/L	35 nmol/L	>150 nmol/L
Monodesethylamodiaquine	47 nmol/L	17 nmol/L	162 nmol/L	>80 nmol/L
Dihydroartemisinin	1.1 nmol/L	2.5 nmol/L	3.0 nmol/L	>10.5 nmol/L
Atovaquone	13.3 nmol/L	4.1 nmol/L	3.6 nmol/L	>350 nmol/L
Cycloguanil	70 nmol/L	<10 nmol/L	1191 nmol/L	>500 nmol/L
Pyrimethamine	354 nmol/L	<50 nmol/L	9139 nmol/L	>2,000 nmol/L
Doxycycline	12.8 μmol/L	10.5 μmol/L	13.5 μmol/L	>35 μmol/L
Azithromycin	24 μmol/L	48 μmol/L	39 μmol/L	ND
Clindamycin	39 μmol/L	108 μmol/L	126 μmol/L	ND

*_P. falciparum_ strains 3D7 and W2 were used as controls. ND, not determined.

involved in chloroquine resistance (K76T), and the dihydroopteroate synthase gene, which encodes the sulfadoxine target (A437G), identified the resistant allele in our isolate (*6*). There was no mutation in codon 268, which encodes the atovaquone target (*4*). The isolate had only 1 copy of the *P. falciparum* multidrug resistance (*Pfmdr1*) gene and a mutation in codon 184, which suggested in vitro susceptibility to mefloquine (*7*). Amplification of DNA from parasites obtained on day 0 and preserved on fixed and stained thin blood films by a modification of the procedure of Edoh et al. (*8*) was not successful.

Conclusions

Quinine remains a reliable treatment for patients with complicated or severe *P. falciparum* malaria outside southern Asia. Clinical failure with quinine used alone or in combination with clindamycin is common in Africa. In our case-patient, a correlation between the results of the in vivo and in vitro assessments was demonstrated at day 27. Because of the lack of reliable data on the correlation between quinine IC_{50} and clinical failure, arbitrary IC_{50} cutoff values were chosen for in vitro quinine resistance (300 nmol/L, 500 nmol/L, or 800 nmol/L) (*9*).

Quinine resistance appears to share common characteristics with chloroquine resistance. It is associated with mutations in the *pfmdr1* (*10*) and *pfcrt* (*11*) genes. Nevertheless, the mechanism of quinine resistance is still unknown. In addition to the *pfmdr1* and *pfcrt* genes, other genetic polymorphisms such as microsatellite length variations in the *P. falciparum* sodium/hydrogen exchanger (*pfnhe-1*) gene (*5*) and mutations in the *P. falciparum* multidrug resistance protein gene may contribute to quinine resistance (*12*).

We report an association of clinical failure of quinine treatment with an IC_{50} of 829 nmol/L, a mutation in codon 76 of the *pfcrt* gene, and an ms4760–18 profile for *pfnhe-1* composed of 2 DNNND repeats. Isolates of *P. falciparum* with ≥2 DNNND repeats may be associated with reduced susceptibility to quinine. Henry et al. (*5*) reported that 2 DNNND repeats were associated with quinine IC_{50} values ranging from 300 nmol/L to 700 nmol/L, and that 3 repeats were associated with an IC_{50} >600 nmol/L. However, the 3 strains with IC_{50}s >800 nmol/L had ≥2 DNNND repeats (*6*). Our results are consistent with these data.

P. falciparum resistance levels may differ depending on malaria transmission and drug pressure. Data from Senegal are fragmentary and were obtained by in vitro susceptibility studies conducted with isolates reported to have decreased in vitro susceptibility to quinine (*6*). Our patient had traveled to Dielmo, Senegal, where in vitro surveillance of antimalarial drug susceptibility has been conducted since 1996. During 1996–2005, the overall prevalence of isolates with IC_{50} >800 nmol/L for quinine was <6%: 1% in 1996, 4% in 1997, 0% in 1998, 6% in 1999, and 0% in 2005 (*13*). Quinine was used for 96.4% of the treatments administered in Dielmo during 1990–1995 (*14*). This drug has since been replaced by chloroquine, sulfadoxine-pyrimethamine, and artemisinin-based combination therapies.

We report a patient with clinical failure associated quinine resistance in a traveler to Senegal. Our results are consistent with those of a recent review of the Uganda Malaria Surveillance Project that reported a higher risk for selecting quinine-resistant parasites associated with a 7-day quinine treatment course (*15*). Thus, resistance to quinine should be monitored in West Africa. Although such clinical failure of therapy is rare, increased vigilance is required during treatment follow-up, and surveillance of the parasite population should also be increased.

Dr Pradines is a senior researcher at the Research Unit in Parasitological Biology and Epidemiology of the Institute for Tropical Medicine of the French Army, Le Pharo, Marseille, France. His primary research interests are the epidemiology and population genetics of malaria.

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Putative New Lineage of West Nile Virus, Spain

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To ascertain the presence of West Nile virus (WNV), we sampled mosquitoes in 2006 in locations in southern Spain where humans had been infected. WNV genomic RNA was detected in 1 pool from unfed female *Culex pipiens* mosquitoes. Phylogenetic analysis demonstrated that this sequence cannot be assigned to previously described lineages of WNV.

West Nile virus (WNV) has been described in Africa, Europe, the Middle East, Asia, Australia, and, most recently, the Americas. Over the last few years, many reports about WNV have been published after the outbreaks in Romania, Morocco, Italy, Russia, and Israel, but especially with the introduction and spread of the virus in the Americas. Currently, the virus has a wide geographic distribution, and WNV infection is considered an emerging zoonosis (*1*).

Although only WNV lineage 1 is present in the Americas, ≥5 lineages of the virus seem to circulate in the Old World (*2*). In 2008, several countries in Europe reported WNV activity due to different lineages. WNV lineage 1 was isolated from horses and birds in northern Italy, and WNV infection was described in 6 persons (*3*). The Austrian veterinary authorities reported 2 outbreaks of WNV in wild birds, 1 in northern Austria, and 1 in the region of Vienna. The virus isolated from these birds, sparrow hawks, was WNV lineage 2 and was very homologous to 2 strains previously found in goshawks in Hungary in 2004 and 2005. These reports represented the emergence of a WNV lineage 2 strain outside Africa for the first time (*4*). Migratory birds that overwintered in central Africa may have recently introduced this exotic strain in the wetlands of different eastern European countries. Consequently, this neurotropic, exotic WNV strain may become a resident pathogen in Europe with public health consequences.

A new lineage of WNV (named Rabensburg virus), of as yet unknown human pathogenicity, was isolated from *Culex pipiens* mosquitoes in 1997 and 1999 on the Czech Republic–Austria border, only a few hundred kilometers from the region where WNV emerged in Hungary (*5*). The Rabensburg isolate 97–103, obtained from *Cx. pipiens* mosquitoes (1997) in Czech Republic (*6*), and LEIVKrnd88–190, isolated from *Dermacentor marginatus* ticks in a valley in the northwestern Caucasus Mountains in 1998 (*7*), have been proposed to be novel variants of WNV. These isolates are genetically different from viruses of lineage 1 and 2 and have been proposed as members of lineages 3 and 4, respectively. Moreover, 2 other related viruses show no clear relationships with WNV, the strain KUN MP502–66 from Malaysia, and Koutango (KOUV), an African virus, with poor statistical support for clustering with either of the WNVs, which suggests that they represent 2 single-isolate lineages (*8*).

Previous serologic surveys conducted with small rodents and humans in different areas of Spain have shown evidence of WNV circulation (*9*). Although no neurologic illness outbreaks have been documented in Spain, recent studies indicate that WNV is circulating in the southern part of the country, close to the areas of the recent foci in Portugal and Morocco. This part of Spain contains several wetlands, which have high densities of migratory birds and mosquitoes. WNV activity has been reported in this region on the basis of serologic surveys in birds, horses, and humans (*10–12*). Moreover, the first clinical case of WNV infection in Spain was reported in 2004 in a patient visiting southwestern Spain (*13*), and WNV lineage 1 was detected and further isolated in free-living and captive Spanish golden eagles in south-central Spain (*14*). Following up these results, we collected mosquito samples especially from areas from which positive serum samples had been obtained to look for WNV in its vector.

The Study

The area of study included 2 wetlands: Marismas del Odiel (tidal marshes) and Doñana (freshwater marshes), both located in southwestern Spain. Mosquitoes were captured in 2006 with U.S. Centers for Disease Control and Prevention light traps supplied with CO_2 and with gravid traps, which were used in the field during the late afternoon and retrieved the following morning. Mosquitoes were pooled by species, sex, collecting site, and date. The number of mosquitoes per pool ranged from 1 to 50. Mosquitoes/pools were homogenized in a range of 500–700 μL of minimal essential medium supplemented with 200 U/mL of antimicrobial drugs (penicillin/streptomycin) and 10% of fetal bovine serum and then were stored at –80°C until

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DOI: 10.3201/eid1603.091033

Table 1. Mosquitoes collected and tested for flavivirus in Huelva, Spain, 2006

Species	No. pools	No. positive pools
Anopheles algeriensis	6	0
An. atroparvus	21	0
An. claviger	1	0
Anopheles sp.	1	0
Coquillettidia richiardii	2	0
Ochlerotatus caspius	491	33
Oc. detritus	71	1
Oc. geniculatus	2	0
Ochlerotatus sp.	2	0
Culex modestus	131	1
Cx. perexiguus	54	0
Cx. pipiens	457	3
Cx. theileri	308	151
Culex sp.	28	2
Culiseta annulata	13	0
Cs. longiareolata	51	0
Cs. subochrea	1	0
Culiseta sp.	1	0
Total	1,641	191

they were tested for flavivirus. The homogenate was centrifuged at 13,000 rpm for 5 min at 4°C, and the screening was performed with a generic nested reverse transcription PCR (*15)* to detect flavivirus genome.

This study comprised 35,424 mosquito specimens grouped in 1,641 pools and representing 14 species (Table 1). Approximately 11% of the pools (191) showed a positive result for flavivirus amplification. However, WNV was identified in only 1 (pool HU2925/06). The pool contained 50 unfed *Cx. pipiens* complex females, captured in June 2006 in Palos de la Frontera (Huelva; latitude 37°12′41.76″N; longitude 6°55′9.58″W).

A fragment of 1,813 nt from the nonstructural protein 5 (NS5) gene from this WNV genome (GenBank accession no. GU047875) was amplified by using 3 WNV-specific nested-PCRs designed in this study. The phylogenetic analysis resulted in a tree in which, as expected, this sequence fell under the branch of WNV, with a value of certainty of 100% (Figure, panel A). A common evolutionary branch between the Spanish strain and lineage 4 (99% certainty) can be observed, and both strains seem related to lineage 3. Sequence differences observed between HU2925/06 and other strains of WNV are shown in Table 2. The minor genetic distance was obtained for lineage 4 and the highest for lineage 5. To confirm that the sequence detected in Spain did not correspond to those of the isolates KUN MP502–66 and KOUV, we analyzed part of the genome sequence of both viruses, and partial sequences showed that these viruses cluster into a distinct genetic lineage (Figure, panel B). The sequence data for KOUV was retrieved from GenBank (strain Koutango DakArD1470, accession no. AF013384), and the partial sequence for KUN MP502–66 was obtained in this work amplifying part of the NS5 gene (GenBank accession no. GU047874).

To isolate the virus, the positive pool was diluted 1:20 in the minimal essential medium, and 200 μL were injected onto C6/36 (*Aedes albopictus* cells), RK-13 (rabbit kidney cells), and Vero (African green monkey cells) monolayer cells grown at a constant temperature for each cell line (33°C, 37°C, and 37°C, respectively). Cell cultures were incubated under the same conditions for 7 days, and 3 blind passages were carried out. Signs of cytopathic effect were checked daily, and the culture supernatants were tested by

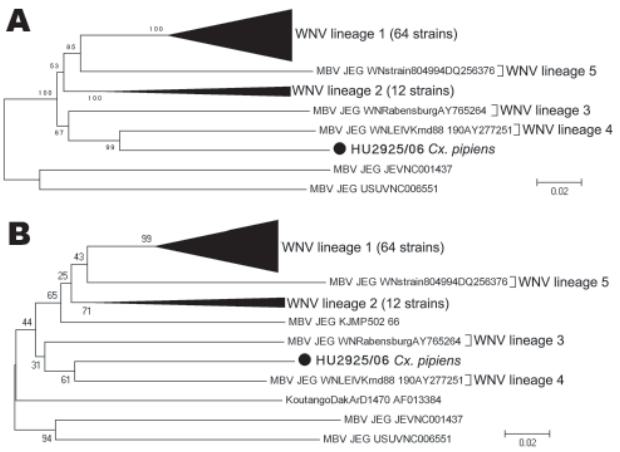

Figure. Phylogenetic tree of 79 WNV isolates by the neighbor-joining method and distance-p model on MEGA3.1 (www.megasoftware.net/mega_dos.html). Bootstrap values correspond to 1,000 replications. A) Analysis of a 1,813-nt fragment of the nonstructural protein 5 (NS5) gene. B) Analysis of the 800-nt fragment of the NS5 gene. KOUV (strain DakArD1470, AF013384) and Malaysia (strain KUN MP502–66, GU047874) (**boldface**) were also used to obtain this tree. Scale bars indicate nucleotide substitutions per site. WNV, West Nile virus; nt, nucleotide; MBV, mosquito-borne viruses; JEG, Japanese encephalitis group; JEV, Japanese encephalitis virus; USUV, Usutu virus. Viruses used in the phylogenetic study (GenBank accession nos.): WNV lineage 1 (AY712948, AY712947, AY490240, AY278442, AY278441, AY277252, AF404757, AF404756, AF404755, AF404754, AF404753, AF481864, AY603654, AY646354, AY289214, AY795965, AY842931, AY660002, AF196835, DQ164206, DQ164202, DQ164197, AF260969, AF260968, AF260967, DQ211652, DQ164204, DQ164200, DQ164201, AF533540, DQ005530, DQ118127, DQ164205, DQ164203, DQ164199, D00246, DQ164196, DQ080058, DQ080054, DQ080055, DQ080056, DQ164193, DQ164186, DQ164195, DQ164191, DQ164205, DQ080053, AY848696, AB185917, DQ080052, AB185914, DQ080051, DQ164189, AF404756, DQ164190, AY712945, AY712946, DQ080059, DQ164188, DQ164187, DQ164192, AF404757, AY277252, AY274505); WNV lineage 2 (DQ116961, DQ318019, M12294, EF429200, AY532665, EF429198, EF429199, EF429197, NC001563, AY688948, DQ176636, DQ318020); WNV lineage 3 (AY765264); WNV lineage 4 (AY277251); WNV lineage 5 (DQ256376); Spanish WNV (HU2925/06, GU047875); JEV: (NC001437); and USUV (NC006551) (as an outgroup).

Table 2. Sequence differences between HU2925/06 and other strains representing previously described West Nile virus lineages or related flaviviruses*

	Nucleotide difference, %						Amino acid difference, %		
Lineages	1a	1b	2	3	4	5	HU2925/06	JEV	USUV
1a	–	1.1	5	6.7	7.8	6	8.3	17.8	16.5
1b	11.1	–	4.8	6.8	7.6	6	8.1	17.9	16.7
2	20	21.4	–	5.5	6.1	6.7	7.7	18.5	17.6
3	21.6	22	20.8	–	6.8	7.8	8.6	18.1	18.1
4	21.8	22	22	22.6	–	9.5	5	19.6	19.1
5	19.6	20.2	21.3	22.3	23.4	–	10.9	18.4	17.1
HU2925/06	22.5	22.4	22.2	22	18.3	23.4	–	19.1	19.2
JEV	27.3	28.2	27.1	26.9	29	27.7	28	–	14.3
USUV	26.1	26.8	27.8	29.1	28.1	27.6	28.2	25	–

*Values of nucleotide and amino acid differences were calculated by p distance and multiplied by 100. JEV, Japanese encephalitis virus; USUV, Usutu virus.

reverse transcription–PCR. Neither cytopathic effect nor amplification was obtained. No virus was isolated from any of the 3 cell cultures.

Conclusions

The phylogenetic analysis performed on a 1,813-nt fragment of the NS5 gene clearly shows that the sequence recovered in Spain grouped within the branch of WNV with high values of certainty (100%). The tree topology shows a common evolutionary branch between the Spanish WNV genome (HU2925/06) and lineage 4, which clusters close to lineage 3. The lineages 3 and 4 were detected recently in Europe (1997 and 1998, respectively), and they have not been previously associated with natural disease in vertebrates. In addition, the phylogenetic analysis performed on 800 nt fragments of the NS5 gene indicated that the Spanish strain was not the same that KUN MP502–66 and KOUV, and that KUN MP502–66 seems to be a different lineage.

This report and the recent description of WNV lineage 1 in wild birds (*14*) demonstrate the circulation of both WNV lineages in Spain. This finding should lead to the analysis of serologic evidence of WNV infections in birds, horses, and humans in Spain and surrounding countries, where the highly pathogenic WNV strains sporadically cause clinical infections. An explanation for the high WNV seroprevalence levels found in birds, horses, and humans in the absence of neurologic disease in Spain could be that this new lineage infects birds and protects them from most pathogenic strains of WNV.

Acknowledgments

We are grateful to Gema Rojo, Rubén Villalba, Azucena Sánchez, Tomás Mayoral, and Montserrat Agüero for their help and technical support. We also thank Hervé Zeller for providing an aliquot of virus from Malaysia (isolate KUN MP502-66).

This work is part of the multidisciplinary network Enfermedades Viricas Transmitidas por Artrópodos y Roedores (funded by Instituto de Salud Carlos III [ISCIII] G03/059). It has been supported in part by the European Commission (contract 010284-2, Emerging Diseases in a Changing European Environment Project contribution EDEN0157), and grants FIS PI07/1308, Red de Investigacion de Centros de Enfermedaes Tropicales RD06/0021, and the agreement signed between the Institute of Health Carlos III and the Spanish Ministry of Health and Social Policy for the surveillance of imported viral hemorrhagic fevers.

Dr Vázquez is a postdoctoral researcher at the Spanish Institute of Health Carlos III. Her research interests include emerging arboviruses transmitted by mosquitoes, especially flaviviruses.

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Venezuelan Equine Encephalitis and 2 Human Deaths, Peru

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Studies have suggested that enzootic strains of Venezuelan equine encephalitis (VEE) subtype ID in the Amazon region, Peru, may be less pathogenic to humans than are epizootic variants. Deaths of 2 persons with evidence of acute VEE virus infection indicate that fatal VEEV infection in Peru is likely. Cases may remain underreported.

Venezuelan equine encephalitis (VEE) is an emerging zoonotic disease in the Amazon region of Peru. After dengue, it is considered the second most important arboviral disease in Peru. Most human infections with VEE virus (VEEV) are caused by subtype ID (*1*–*5*), and within subtype ID, 6 genotypes have been described (*6*). In Peru, the Colombia/Venezuela, Panama/Peru, and Peru/Bolivia genotypes have been identified among VEEV subtype ID isolates (*1*,*5*,*7*). Epidemiologic investigations have failed to detect neurologic disease or deaths among >200 VEE cases in this country (T.J. Kochel, unpub. data). Only 2 fatal cases with VEEV subtype ID have been reported, both in Panama (*6*,*8*). In contrast, fatal cases with neurologic complications (estimated mortality rate 0.7%) have been described regularly for human outbreaks caused by VEEV subtypes IAB and IC (*9*–*12*). On the basis of these reports, it has been suggested that enzootic VEEV strains in Peru may be less pathogenic to humans than the epizootic variants (*13*). However, only 200 cases identified in Peru may not be enough to make such an assertion.

We recently described a severe infection in a 3-year-old boy who had VEEV subtype ID (*14*). Here we describe 2 fatal infections in persons with evidence of acute VEEV infection in Peru. One patient had confirmed subtype ID.

The Study

In 2000, the Naval Medical Research Center Detachment (NMRCD) and the Ministry of Health of Peru established a passive surveillance study to determine arboviral causes of febrile illness (protocol NMRCD.2000.0006). Patients with acute, undifferentiated febrile illness of <7 days were invited to enroll, and demographic and clinical information was obtained at the time of enrollment. Blood samples were obtained and assayed by virus isolation, and convalescent-phase samples were obtained 10 days to 4 weeks later for serologic studies.

Patient 1 was a 7-year-old girl from San Benito, a rural community near Yurimaguas city (Figure 1), who on June 19, 2006, was noted to have nasal congestion, sneezing, chills, malaise, myalgia, abdominal pain, and fever followed by several episodes of watery, nonbloody feces, and vomiting. Her condition rapidly worsened, and she began having tonic–clonic seizures. The next day the involuntary movement and vomiting stopped; however, other signs worsened and she became somnolent and prostrate. Later that day she was admitted to the Hospital Santa Gema of

Figure 1. Selected sites in Peru of passive surveillance study to determine arboviral causes of febrile illness in Peru, established in 2000 by Naval Medical Research Center Detachment and the Ministry of Health of Peru (protocol NMRCD.2000.0006). Sites shown include those of 2 patients with evidence of acute Venezuelan equine encephalitis virus infection. Shaded area is Titicaca Lake.

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DOI: 10.3201/eid1603.090970

Yurimaguas with fever, dehydration, stupor, and signs of respiratory distress along with tonic–clonic seizures. Temperature was 40°C (104°F), blood pressure 90/50 mm Hg, respiratory rate markedly increased, and heart rate 132 beats/min. Distal cyanosis, nasal flaring, rhonchi without crackles, and supraclavicular and intercostal retractions were noted, but no jaundice, lymphadenopathy or conjunctival hemorrhage were observed. Hepatomegaly (liver 4 cm under the costal border), stupor, and neck stiffness were also noted. Laboratory test results showed a left shift in the leukocyte count and renal failure (Table 1). Blood smears were negative for malaria parasites. Preliminary diagnoses were sepsis, convulsive status, and respiratory distress. Later that day the patient was considered to have a complicated, febrile, neurologic illness, and a blood sample was sent for advanced analysis at the NMRCD in Lima, Peru, and the National Institute of Health of Peru.

The girl was given supportive therapy with broad spectrum antibiotics, intravenous hydration, oxygen, and anticonvulsive medications (diazepam, phenytoin). On June 21, seizures persisted, and the patient became comatose and later died of respiratory arrest. Arterial blood gasses and electrolytes could not be measured because of the limited capacity of the hospital laboratory. The girl's parents did not consent to lumbar puncture or autopsy.

Examination of the girl's serum in Vero cells identified VEEV, and sequencing and phylogenetic analyses using previously described methods further identified it as subtype ID Panama/Peru genotype (Figure 2) (*15*). On the day the serum was collected (1 day after onset of signs), viremia titer was 1.8×10^4 PFU/mL, similar to titers from other VEEV-infected study patients who did not have neurologic complications (Table 2). The National Institute of Health reported the sample to be negative for leptospiral and rickettsial organisms, according to ELISA immunoglobulin (Ig) M and indirect immunofluorescent assays, respectively. Blood and cerebrospinal fluid cultures for bacteria were not attempted.

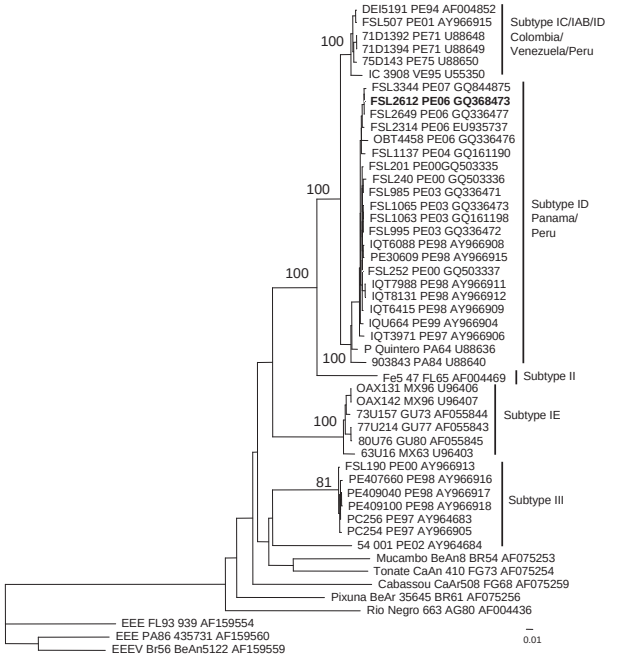

Figure 2. Neighbor-joining phylogenetic tree of Venezuelan equine encephalitis virus (VEEV) complex based on partial sequence of the PE2 segment (nucleotide positions ≈8385–9190 of the VEEV genome). The tree was rooted by using an outgroup of 3 major lineages of Eastern equine encephalitis virus (EEEV). The strain isolated from a 7-year-old girl who died from acute VEEV infection in Peru, June 21, 2006, is in **boldface.** Viruses are labeled by code designation, abbreviated location name, year of isolation (last 2 digits of year only), and GenBank accession numbers of the corresponding sequences. PA, Panama; GU, Guatemala; MX, Mexico; FG, French Guiana; VE, Venezuela; BR, Brazil; AG, Argentina; PE, Peru; FL, Florida. Numbers indicate bootstrap values. Scale bar indicates nucleotide substitutions per site.

Patient 2 was a 25-year-old man from Puerto Maldonado, a city in the department of Madre de Dios (Figure 1). On February 24, 2005, he reported fever, headache, myal-

Table 1. Laboratory test results for 2 patients infected with Venezuelan equine encephalitis virus, Peru*

Testing	Patient no. 1	Patient no. 2		Reference range
Date blood collected	2006 Jun 20	2005 Feb 28	2005 Mar 1	
Hematocrit, %	36	36	22	38–44
Thromocytes, cells/mm^3	233,000	143,750	NM	150,000–450,000
Leukocytes				
Total, cells/mm^3	21,450	3,900	NM	4,500–10,000
Bands, %	7	4	NM	3–5
Segmented cells, %	84	72	NM	55–65
Eosinophils, %	0	0	NM	0.5–4.0
Monocytes, %	0	14	NM	3–8
Lymphocytes, %	9	10	NM	25–35
ESR, mm/h	41	NM	NM	1–15
Creatinine, mg/dL	2.1	9.1	8.8	0.8–1.4
Urea, mg/dL	115	206	238	20–45

*NM, not measured; ESR, erythrocyte sedimentation rate.

Table 2. Venezuelan equine encephalitis virus titers in 10 patients, Peru

Patient code	Age, y/sex	Location	Date of symptom onset	Date of blood collection	Serum titer, PFU/mL	Signs and symptoms						
						Death	Bleed*	Fever	Seizure	GI†	Resp‡	Aches§
Patient 1 FSL2612	7/F	Yurimaguas, Loreto	2006 Jun 19	2006 Jun 20	1.8×10^4	+	–	+	+	+	+	+
FSL2649	32/M	Yurimaguas, Loreto	2006 Aug 7	2006 Aug 8	3.4×10^4	–	–	+	–	+	+	+
IQE1149	14/F	Iquitos, Loreto	2005 Apr 14	2005 Apr 15	1.0×10^3	–	–	+	–	–	–	+
IQE831	8/F	Iquitos, Loreto	2005 Mar 13	2005 Mar 14	3.0×10^2	–	–	+	–	–	–	+
IQE3605	6/M	Iquitos, Loreto	2006 Apr 3	2006 Apr 4	1.0×10^2	–	–	+	–	+	–	+
IQE3741	10/F	Iquitos, Loreto	2006 Apr 21	2006 Apr 24	5.0×10^2	–	–	+	–	+	–	+
IQE5023	7/M	Iquitos, Loreto	2007 Feb 27	2007 Feb 28	1.3×10^4	–	–	+	–	+	–	+
FMD1737	18/M	Puerto Maldonado, Madre de Dios	2007 Dec 17	2007 Dec 19	5.0×10^2	–	–	+	–	–	–	+
FMD1905	21/F	Puerto Maldonado, Madre de Dios	2008 Feb 9	2008 Feb 12	3.0×10^3	–	–	+	–	+	–	+
Patient 2¶	25/M	Puerto Maldonado, Madre de Dios	2005 Feb 24	2005 Feb 28	NM	+	+	+	–	+	–	+

*Epistaxis, bleeding gums, ecchymosis, purpura, hematochezia, or melena.
†Gastrointestinal (nausea, vomiting, diarrhea, or abdominal pain).
‡Respiratory (cough, dyspnea, or cyanosis).
§Arthralgia, myalgia, bone pain, malaise, or prostration.
¶Virus isolation attempts unsuccessful; ELISA immunoglobulin M results positive for Venezuelan equine encephalitis. NM, not measured.

gia, nausea, vomiting, and diarrhea. Because he was from an outlying rural area, he was taken to the local health center where he received intravenous rehydration and partially recovered. The patient stayed at home, but his signs and symptoms persisted. On February 27, jaundice and epistaxis developed, and the local health center referred him to the Santa Rosa Hospital in Puerto Maldonado, where he was admitted on February 28. The patient's status deteriorated quickly; hematuria and hematemesis were followed by liver and renal failure (Table 1), and the patient died on March 1. Postmortem examination found multiple hemorrhages in his lungs, kidneys, and stomach. A serum sample collected at the time of admission was positive for VEEV antibodies, according to ELISA IgM (titer 6,400) (*2*,*3*). The case was presumed to be VEE, although virus isolation attempts were unsuccessful. Serologic assays produced negative results for leptospiral and arboviral diseases, including dengue, Mayaro, yellow fever, Oropouche, and Eastern equine encephalitis.

Conclusions

Patient 1 had no previous history of neurologic disease or poor health. VEEV subtype ID infection (Panama/Peru genotype) was confirmed. Because her viremia titer was similar to titers of other patients who did not have neurologic complications and survived VEEV infection, viremia levels alone may not account for the difference in disease outcome. Although VEEV was isolated from the patient's serum and she met the Centers for Disease Control and Prevention's diagnostic criteria for confirmed VEE (www.cdc.gov/ncphi/disss/nndss/casedef/arboviral_current.htm), we cannot rule out concomitant bacterial meningitis in this patient, who had meningismus and leukocytosis. The limited extent of our diagnostic procedures prevent us from concluding with certainty that VEEV infection was the main cause of death.

Patient 2 had severe hemorrhagic complications. Although an uncommon manifestation of VEEV infection, these complications have been reported elsewhere (*6*,*8*,*14*). To date, only VEEV subtype ID has been isolated in and around Puerto Maldonado (*5*); thus, this patient was probably also infected with this subtype. However, we cannot unequivocally state that the patient died from VEEV infection.

Both fatal cases described in this report were clinically similar to previously reported enzootic and epidemic VEE cases (*1*,*6*,*8*,*9*,*11*,*14*). Initially, both patients had fever, body aches, vomiting, and diarrhea (Table 1) (*1*, *6*,*8*,*9*,*11*), which are also caused by other tropical diseases like dengue. Only some patients, such as patient 1 from Yurimaguas, had neurologic complications (Table 1), which are more commonly observed in children<15 years of age (*6*,*8*,*9*,*11*).

Our surveillance activities were limited to only 8 surveillance sites in Peru (Figure 1), so VEE cases in other areas may remain undiagnosed. Because of the lack of surveillance activities and proper diagnostic capabilities, fatal VEEV infection in Peru is likely; many cases may remain underreported in isolated rural locations where the disease is most common. Additional studies are needed to fully measure the extent and effects of VEE in Peru.

Acknowledgments

We thank Carolina Guevara, Zonia Rios, Roxana Caceda, Vidal Felices, Cristhopher Cruz, Connie Fernandez, Aydee Cruz, and Javier Ignacio Effio for invaluable support in the execution of the study. We also thank the personnel of the Hospital de Apoyo Yurimaguas for supporting our febrile illness surveillance study.

This study was funded by the US Department of Defense Global Emerging Infections Systems Research Program, Work Unit no. 847705.82000.25GB.B0016. The study protocol for Surveillance and Etiology of Acute Febrile Illnesses in Peru was approved by the Naval Medical Research Center Institutional Review Board (protocol NMRCD.2000.0006) in compliance with all applicable federal regulations governing the protection of human subjects.

Dr Vilcarromero is a physician who participates in the Surveillance and Etiology of Febrile Acute Diseases in Peru project, conducted by the NMRCD, Peruvian Ministry of Health, and Cayetano Heredia and San Marcos Universities in Lima. He is responsible for the project in an extensive area in the jungle of Peru, and his work is now based in the city of Iquitos.

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Extensively Drug-Resistant *Mycobacterium tuberculosis* from Aspirates, Rural South Africa

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The yield from aspirating lymph nodes and pleural fluid for diagnosing extensively drug-resistant (XDR) tuberculosis is unknown. *Mycobacterium tuberculosis* was cultured from lymph node or pleural fluid aspirates of 21 patients; 7 (33%) cultures grew XDR *M. tuberculosis*. Additive diagnostic yield for XDR *M. tuberculosis* was found in parallel culture of sputum and fluid aspirate.

Tuberculosis (TB) is the leading cause of death among HIV-infected persons in sub-Saharan Africa (*1*). Drug-resistant TB is an emerging public health threat in HIV-prevalent settings, but diagnosis is challenging because of the severely limited laboratory capacity for culture and drug-susceptibility testing (DST). TB diagnosis for HIV-infected patients is particularly challenging because these patients may be more likely to have smear-negative pulmonary disease or extrapulmonary TB (*2*,*3*). Extrapulmonary TB often is diagnosed by clinical findings, indirect measures (e.g., chemistry and cell count of cerebrospinal or pleural fluid, ultrasound of lymph nodes, or pericardial effusions), or smear microscopy for acid-fast bacilli from aspirated extrapulmonary fluid. However, drug-resistant TB is impossible to diagnose by these methods, instead requiring mycobacterial culture and DST (*4*,*5*).

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DOI: 10.3201/eid1603.091486

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Recognize how concomitant HIV infection can affect the diagnosis of tuberculosis
- Describe procedures and patient characteristics in the current study
- Identify how aspirate cultures can help identify extensively drug-resistant tuberculosis
- Specify the percentage of patients with tuberculosis who were diagnosed with aspirate but not sputum cultures.

Editor

Karen L. Foster, Writer/Editor, Emerging Infectious Diseases. *Disclosure: Karen L. Foster has disclosed no relevant financial relationships.*

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The prevalence of multidrug-resistant and extensively drug-resistant TB (XDR TB) in South Africa has risen exponentially during the past decade. At our rural study site, ≈10% of all TB cases now are drug resistant, and >90% of TB patients are HIV infected (*6*). Death from XDR TB exceeds 80%; most infected persons die before sputum culture and DST results are known (*6*). To improve case detection and decrease diagnostic delay of drug-resistant TB among

patients with suspected extrapulmonary TB, we initiated a program to aspirate lymph nodes and pleural fluid for culture and DST. We quantified the yield of these lymph node and pleural fluid aspirates for diagnosing XDR TB.

The Study

We performed a retrospective cross-sectional study to determine the proportion of patients in whom TB and XDR TB could be diagnosed by culture of fine-needle aspiration of a lymph node or pleural fluid. Additionally, we sought to determine the yield of lymph node and pleural fluid aspiration beyond culture of sputum alone. All patients were eligible who had an aspirate of a noninguinal lymph node or pleural fluid sent for mycobacterial culture and DST during September 1, 2006–December 31, 2008, from the district hospital in rural Tugela Ferry, South Africa. This 355-bed hospital serves 200,000 Zulu persons.

Hospital protocol was for lymph node aspirates to be obtained at the bedside by using sterile technique and large-bore needle at the point of maximal swelling. Pleural fluid was obtained by standard thoracentesis. Care was taken not to introduce air while injecting the fluid specimen into mycobacterial blood culture bottles (BACTEC MycoF-lytic, Becton Dickinson, Sparks, MD, USA). Smear microscopy was not performed on the fluid aspirate. Bottles were transported to the provincial TB referral laboratory in Durban and cultured by using the automated BACTEC 9240 analyzer (Becton Dickinson) in which growth is continuously monitored for 42 days. *Mycobacterium tuberculosis* was confirmed with niacin and nitrate reductase tests. DST of positive cultures was performed by using the 1% proportional method on Middlebrook 7H11 agar for isoniazid (critical concentration, 0.2 μg/mL), rifampin (1 μg/mL), ethambutol (7.5 μg/mL), ofloxacin (2 μg/mL), kanamycin (6 μg/mL), and streptomycin (2 μg/mL) (*7*). XDR TB was defined as *M. tuberculosis* resistant to at least isoniazid, rifampin, ofloxacin, and kanamycin (*8*). Aspirate culture results were compared with sputum culture results if the patient also had a sputum culture performed within 2 weeks of the lymph node or pleural fluid culture. Standard practice was for 1 sputum specimen to be collected for smear microscopy and another specimen to be collected for culture, depending on the patient's ability to expectorate. Sputum was cultured by using the automated BACTEC MGIT 960 system (Becton Dickinson); DST of positive specimens was performed as described above (*9*).

Medical records were reviewed for basic demographic and clinical data, including age, sex, HIV status, antiretroviral therapy, and TB history. The yield of lymph node and pleural fluid aspirates for detecting *M. tuberculosis* and drug resistance was described by using simple frequencies. Incremental yield of aspirate was calculated for patients who had collection for sputum and either lymph node or pleural fluid aspirate. Ethical approval was obtained from the University of KwaZulu-Natal, Yale University, and Albert Einstein College of Medicine.

For 77 patients, either a lymph node (n = 34) or pleural fluid (n = 33) was aspirated for culture and DST during the study period (Table 1). No patient had both pleural fluid and lymph node aspirates performed.

Of the 34 lymph node cultures performed, 12 (35%) grew *M. tuberculosis*, 1 (3%) grew nontuberculous mycobacteria, and 2 (6%) were other bacteria that were not further speciated (Table 1). Of the 12 positive *M. tuberculosis* cultures, 9 (75%) were drug-susceptible *M. tuberculosis*, and 1 (8%) was XDR *M. tuberculosis*; for 2, DST results were missing. Concurrent sputum samples were available for 6 (50%) of the 12 culture-positive *M. tuberculosis* lymph node aspirates: 3 (50%) were concordant with the aspirate culture (2 drug-susceptible and 1 XDR), and 3 (50%) were sputum culture negative.

Of the 33 pleural fluid cultures performed, 9 (27%) grew *M. tuberculosis*, 1 grew *Cryptococcus* sp., and 1 grew another bacterium (Table 1). Of the 9 *M. tuberculosis* culture-positive pleural fluid specimens, 3 (33%) were drug-susceptible and 6 (67%) were XDR. Among these 9 patients, 5 (55%) had concurrent sputum samples available: 3 (60%) were concordant with the aspirate culture (1 drug-susceptible and 2 XDR), and 2 (40%) were sputum culture negative.

From 17 patients, a sputum sample and either a lymph node or a pleural fluid aspirate was collected for culture and DST (Table 2). For 14, at least 1 specimen was culture positive for *M. tuberculosis*, of which 9 (64%) were positive for sputum, 11 (79%) were positive in the lymph node or pleural fluid, and 5 (36%) were positive by fluid aspirate alone, including 2 patients with XDR *M. tuberculosis* (Table 2).

Conclusions

In this study of predominately HIV-infected patients suspected of having extrapulmonary TB, one third of positive *M. tuberculosis* cultures from lymph node or pleural fluid aspirates were XDR. The additive yield for the diagnosis of any TB of these aspirates above sputum culture alone was 36%. Our findings suggest that strategies of solitary sputum culture or reliance on microscopy of non-sputum fluid analysis would miss opportunities to diagnose drug-resistant TB. Parallel culture of sputum and aspirate fluid appears to be of substantial added benefit for diagnosing XDR TB in this setting.

Our study has several limitations. Aspirates were collected on the basis of the attending physician's clinical judgment. Therefore, the yield for *M. tuberculosis* and XDR *M. tuberculosis* may be overestimated, and other factors that may have influenced the physician's suspicion of drug-resistant TB or increased the likelihood of a posi-

Table 1. Patient characteristics and results of aspirate cultures for *Mycobacterium tuberculosis*, South Africa, September 1, 2006–December 31, 2008*

Characteristic	Lymph node	Pleural fluid
Total no. patients	34	33
Median age, y (IQR)	31 (25–39)	33 (28–39)
Female sex, no. (%)	18 (53)	21 (64)
HIV status, no. (%)		
Positive	30 (88)	24 (73)
Negative	1 (3)	1 (3)
Unknown	3 (9)	8 (24)
Median CD4 cells/mm^3 (IQR)	128.5† (84–375)	207‡ (118–334)
Receiving ARVs,§ no. (% of HIV-infected)		
Yes	14 (47)	13 (54)
No	13 (43)	10 (42)
Unknown	3 (10)	1 (4)
Median duration on ARVs, wk (IQR)	8.4¶ (2.4–21.1)	10.5# (5.8–55.1)
History of prior TB, no. (%)	8 (24)	9 (27)
Receiving TB treatment,§ no. (%)	20 (59)	18 (55)
Median duration of TB treatment, wk (IQR)	12** (4–16)	6†† (3–14)
Positive culture results, no. (%)		
M. tuberculosis	12 (35)	9 (27)
Drug-susceptible TB, no. (% of *M. tuberculosis*)	9 (75)	3 (33)
XDR TB, no. (%)	1 (8)	6 (67)
Missing drug susceptibility results, no. (%)	2 (17)	0
Nontuberculous mycobacteria	1 (3)	0
Cryptococcus sp.	0	1 (3)
Other bacteria	2 (6)	1 (3)

*IQR, interquartile range; ARVs, antiretroviral drugs; TB, tuberculosis; XDR TB, extensively drug-resistant TB.
†Available for 26 HIV-infected patients, excluding CD4% for 1 infant (36%).
‡Available for 18 HIV-infected patients.
§At time of aspirate collection.
¶Available for 10 of 14 patients receiving ARVs.
#Available in 12 of 13 patients receiving ARVs.
**Available for 9 patients.
††Available for 12 patients.

tive aspirate are not known without additional prospective study. It is also not possible to comment on the true incremental yield after comparing with sputum culture; sputum was not collected from all patients, nor were the reasons for lack of collection documented.

Nonetheless, as co-infection with HIV and TB increases in sub-Saharan Africa, the number of persons with extrapulmonary TB, both drug-susceptible and drug-resistant, is anticipated to rise (*10*,*11*). Therefore, diagnostic algorithms for extrapulmonary TB must consider the critical importance of extrapulmonary fluid culture and DST for diagnosis of drug-resistant TB, particularly in HIV-infected persons. Furthermore, this study highlights the need to validate novel diagnostic tests for *M. tuberculosis* drug resistance on nonsputum fluids.

Dr Heysell was supported by the Burroughs Wellcome Fund and the American Society of Tropical Medicine and Hygiene. Drs Shah and Gandhi are supported by the Doris Duke Charitable Foundation Clinical Scientist Development Awards and President's Emergency Plan for AIDS Relief (PEPFAR). Dr Friedland is supported by the Gilead Foundation and PEPFAR. Additional support for this work was provided by the Howard Hughes Medical Institute and Irene Diamond Fund. No supporting organization had any role in the study.

While conducting the study, Dr Heysell was a Burroughs Wellcome Fund/American Society of Tropical Medicine and Hygiene postdoctoral fellow in tropical infectious disease at Yale University and living in Tugela Ferry, South Africa. He is currently a fellow in infectious diseases and international health at

Table 2. Comparison of culture yield for *Mycobacterium tuberculosis* in patients with collection of sputum and either lymph node or pleural fluid aspirate, South Africa, September 1, 2006–December 31, 2008*

	Lymph node, n = 9				Pleural fluid, n = 8			
Result from sputum	DS TB	XDR TB	No DST	No growth	DS TB	XDR TB	No DST	No growth
DS TB	2				1			1
XDR TB		1				2		2
No growth	1		2	3		2		

*DS TB, drug-susceptible tuberculosis; XDR TB, extensively drug-resistant tuberculosis; DST, drug susceptibility testing.

the University of Virginia, with research interests in the epidemiology, diagnosis, and susceptibility testing of drug-resistant tuberculosis.

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Parvovirus 4–like Virus in Blood Products

Jozsef Szelei, Kaiyu Liu, Yi Li, Sandra Fernandes, and Peter Tijssen

Porcine plasma and factor VIII preparations were screened for parvovirus 4 (PARV)–like viruses. Although the prevalence of PARV4-like viruses in plasma samples was relatively low, viruses appeared to be concentrated during manufacture of factor VIII. PARV4-like viruses from human and porcine origins coevolved likewise with their hosts.

In 2005, a previously unknown virus, parvovirus 4 (PARV4), was detected in a plasma sample from a hepatitis B–positive injection drug user (IDU) (*1*). Although PARV4 was subsequently detected in plasma from healthy donors, its prevalence is higher in samples from IDUs, AIDS patients, and hepatitis C virus–infected persons (*2*,*3*). In recent serologic studies, 67% of HIV-infected IDUs had antibodies to PARV4, whereas non-IDU controls were seronegative (*4*) This increased prevalence in IDUs and persons with hemophilia most likely reflects parenteral transmission of the virus (*4*,*5*). Furthermore, PARV4 was frequently detected in human coagulation factor concentrates prepared from older plasma samples (*6*). The lower detection frequency in current concentrates may be due to exclusion of high-risk batches, e.g., from IDU or hepatitis C virus–infected persons during plasma collection, and to improved purification methods. The presence of PARV4 in plasma suggests a viremic phase enabling spread of the virus to different organs. Even though recent studies by Kleinman et al. indicate that parvovirus B19 is not readily transmitted to susceptible hosts by blood component transfusion, similar evaluation of PARV4 transmission will be invaluable in assessing the need to routinely screen for this emerging virus (*7*).

PARV4 contains a 5-kb single-stranded DNA genome with inverted terminal repeats and a large open reading frame (ORF) in each half of the genome coding for nonstructural (NS) protein and structural protein, respectively. PARV4-like viruses form a separate cluster among the parvoviruses (*1*,*8*). Three genotypes of human PARV4 parvoviruses with ≈93% nucleotide sequence identity have been described. The sequence of genotype 1 (PARV4-g1) is highly conserved, whereas that of genotype 2 (PARV4-g2 [formerly PARV5]) is somewhat more diverse. PARV4-g2 is found mostly in older coagulation factor concentrates (1960s–1980s), suggesting that genotype 1 emerged recently (*6*,*8*). A third genotype (PARV4-g3) was isolated from persons in sub-Saharan Africa (*9*). Additionally, PARV4-like viruses with a 60%–65% nucleotide identity were recently identified at high frequencies in porcine and bovine tissue samples in People's Republic of China (*10*).

In this study, porcine plasma samples and factor VIII (FVIII) concentrates used by persons with hemophilia who have autoimmune antibodies against human FVIII were investigated for PARV4-like viruses. We then determined the degree of identity of these isolates with the human virus.

The Study

Plasma samples from healthy pigs were collected in Great Britain in 2001. Initially, these samples were tested for PARV4-like viruses by using previously described degenerate PCR primers (*10*). DNA was extracted from samples by using the High Pure DNA Isolation

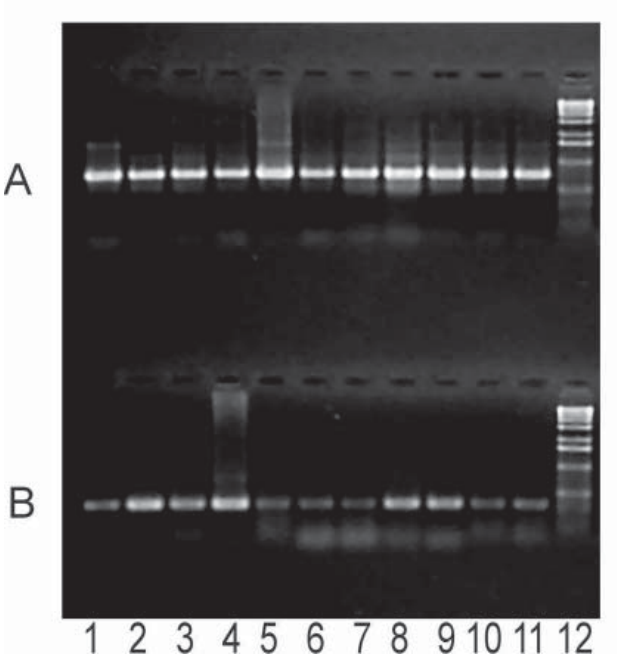

Figure 1. Parallel PCR amplification of PARV4-like (A) and PPV (B) by using purified DNA from clotting FVIII preparations. The results of this PCR usually suggested a higher PARV4 load despite the higher efficiency of the PPV PCR (J. Szelei and P. Tijssen, unpub. data). This finding was confirmed with the quantitative MIMIC PCR method for PPV (*11*). Numbers indicate different lots of FVIII prepared in 1:1994A, 2:1994B, 3:1996A, 4:1996B, 5:1999, 6:2000A, 7:2000B, 8:2001A, 9:2001B, 10:2001C, 11:2001D, and 12: DNA marker (1-kb ladder; Invitrogen, Carlsbad, CA USA). PARV4, parvovirus 4; PPV, porcine parvovirus; FVIII, factor VIII.

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DOI: 10.3210/eid1603.090746

Table 1. Percentage diversity of genome sequences of PARV4-like viruses*†

Genotype	PARV4-p	PHoV	BHoV	PARV4-g1	PARV4-g2
PARV4-p	98–99				
PHoV	97–98	98–99			
BHoV	62	62	99		
PARV4-g1	58	58	60	98–100	
PARV4-g2	58–59	58	59–60	91–92	96–99
PARV4-g3	58	58	60	92	91–92

*PARV4, parvovirus type 4; PARV4-p, porcine PARV-4; PHoV, porcine hokovirus; BHoV, bovine hokovirus; PARV4-g1, PARV4 genotype 1; PARV4-g2, PARV4 genotype 2; PARV4-g3, PARV4 genotype 3.
†Pairwise sequence comparisons were performed by using the ClustalW program (www.ebi.ac.uk/Tools/clustalw) as described in Figure 2 and percentages of sequence identities were calculated. Nucleotide sequences representing the equivalent regions (position 248–5088, numbered according to the PARV4 sequence NC_007018) were used to align the DNA fragments.

Kit (Roche Applied Science, Roche Diagnostics Canada; Laval, Quebec, Canada). Only 3 of the 98 plasma samples contained detectable amounts of PARV4-like viruses. To further study these porcine viruses, we obtained nearly full-length genomes from overlapping PCR fragments. Primers designed for these PCRs were PrS1: 5′-CCACACCTACCTCGCCTATAAGAATCAG-3′; PrAS1: 5′-CTCCACTTGTTCAGCACGGGATCC-3′; PrS2: 5′-CCACGAGCTGGAAGTCTTTA-3′; PrAS2: 5′-GGAGTCCGCATACCCATAACAGGCTG-3′; PrS3: 5′-GTGTACCGCAGTGGGAGCCATG-3′; and PrAS3: 5′-TTCTGGGCAACCCACTGATCAGAAGG-3′. The nearly full-length clones were sequenced by primer-walking. Genomic analysis confirmed that these viruses were related to the PARV4 viruses and were close relatives of the recently identified porcine hokoviruses (PHoVs) (*10*).

We also confirmed the moderate frequency of PARV4-like viremia in the previously tested pig plasma samples with a more sensitive PCR assay by using specific primers PrS4 (5′-AGTTACGGGGGACCGCTACAGTG-3′) and PrAS3. In contrast, examination of 11 commercial clotting FVIII preparations showed that all of these independent lots contained substantial amounts of PARV4-like parvovirus, whereas the level of porcine parvovirus DNA was generally lower in the corresponding samples (Figure 1). Similar to the plasma samples, long overlapping PCR frag-

Table 2. Analysis of relationships among the protein sequences of PARV4-like viruses*†

Sequence	PARV4-p	PHoV	BHoV	PARV4-g1	PARV4-g2	PARV4-g3
PARV4-p						
NS	99–100	(99)	(80)	(68)	(68)	(68)
VP	99–100	(99)	(79)	(77)	(78)	(77)
SAT	100					
PHoV						
NS	97–98	98–99	(79)	(68)	(68)	(68)
VP	99	99	(79)	(77)	(77)	(77)
SAT	98–100	98–100				
BHoV						
NS	67–68	67	99	(70)	(70)	(70)
VP	66	66	NA	(78)	(78)	(78)
SAT	79	79	100			
PARV4-g1						
NS	53–55	53–54	56–57	96–99	(99)	(98)
VP	65	65	65	99	(99)	(98–99)
SAT	59	59	59	100		
PARV4-g2						
NS	54–55	53–54	56	96–97	98–99	(98)
VP	65	65	64–65	98	98–99	(98)
SAT	59	59	59	100	100	
PARV4-g3						
NS	54	53–54	56	96–97	96–97	NA
VP	65	65	64	98	97–98	NA
SAT	59	59	59	100	100	

*PARV4, parvovirus type 4; PARV4-p, porcine PARV-4; PHoV, porcine hokovirus; BHoV, bovine hokovirus; PARV4-g1, PARV4 genotype 1; PARV4-g2, PARV4 genotype 2; PARV4-g3, PARV4 genotype 3; NS, nonstructural protein; VP, viral protein; NA, no alignment; SAT, small alternatively translated proteins.
†Numbers indicate percentages of amino acid sequence identity; numbers in parentheses indicate percentages of amino acid similarity (preserved physicochemical properties). Sequence similarity was not calculated for the SAT proteins, because of their relatively smaller size. When only 1 sequence was available (e.g., VP of BHoV), no alignment was performed.

ments were amplified from the FVIII preparations to obtain nearly full-length sequences. Their analysis provided information about the evolution of PARV4-like viruses, during nearly a decade, in pigs. Sequence data were registered by GenBank (accession nos. Cl2001A: FJ982246; Cl2001B: FJ982247; Cl2001C: FJ982248; F8–1994A: FJ982249; F8–1994B: FJ982250; F8–1996A: FJ982251; F8–1996B: FJ982252; F8–1999: FJ982253; F8–2000A: FJ982254; and F8–2000B: FJ982255). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 4 (*12*).

The genomes of these newly isolated PARV4-like viruses were similar to the PHoVs previously identified in Hong Kong Special Administrative Region, People's Republic of China. Although, these new isolates showed some diversity (98%–99% identity), they differed somewhat more from the PHoVs (97%–98% identity). The viral protein (VP)-ORF was highly conserved (99%), whereas the NS-ORF showed more diversity (97%–98%). Genomic and protein-coding sequences were also compared with other PARV4-like viruses (Tables 1, 2). Phylogenic analysis using neighbor-joining and maximum parsimony methods demonstrated that PHoVs grouped together, whereas PARV4-like sequences from FVIII prepared at different times were less uniform (Figure 2). Older FVIII PARV4 contaminants (especially from 1994) were related more closely to the bovine hokoviruses (BHoVs) and to PARV4-g2. Finally, analysis of the newly identified virus genomes

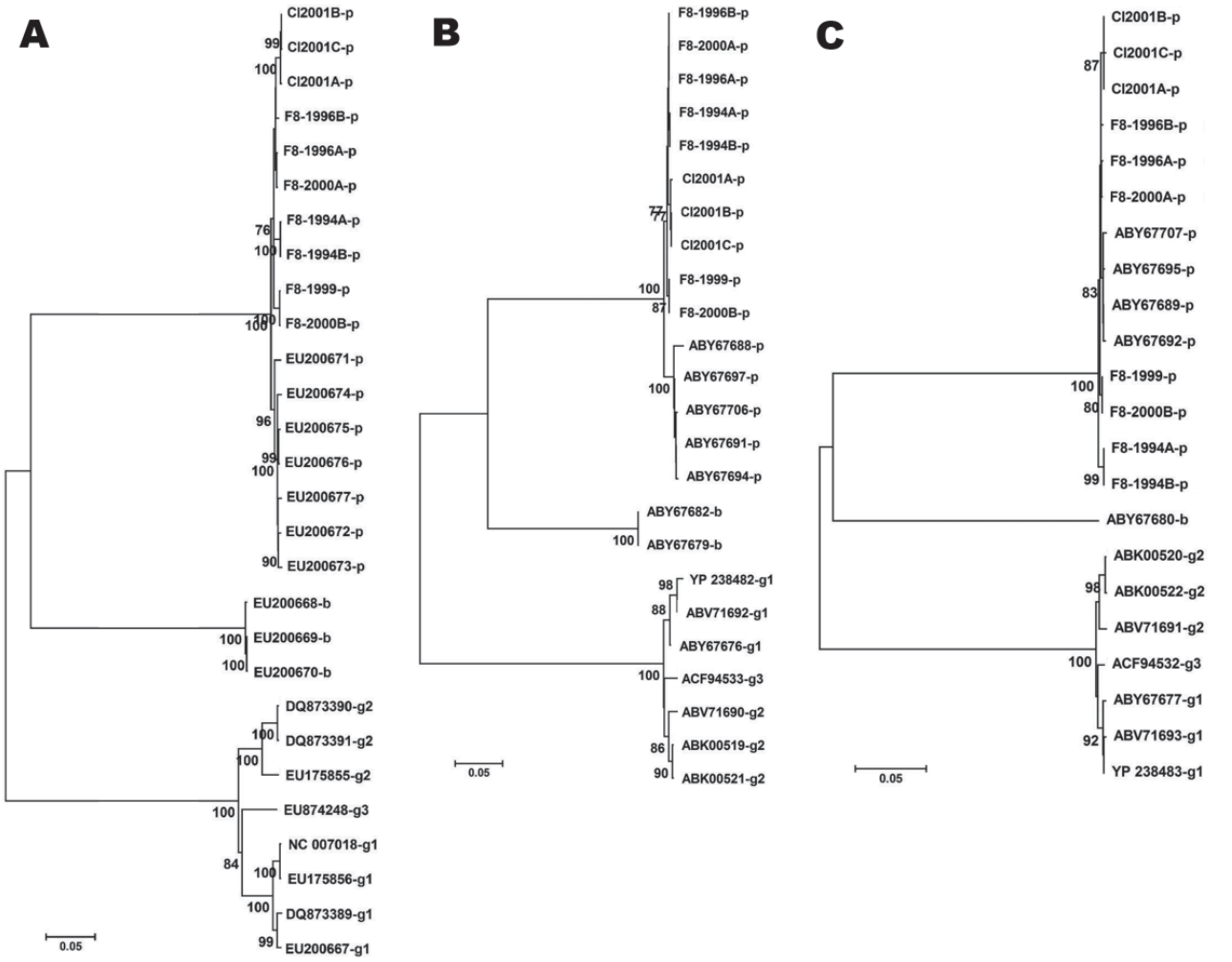

Figure 2. Construction of phylogenic trees for newly identified porcine viruses and comparison with previously identified prototype parvovirus 4 (PARV4)–like sequences. Sequences of other PARV4-like viruses indicated by the accession numbers were obtained from GenBank, and their origins are marked by letters (p, porcine; b, bovine; PARV4-g1, g2, g3, human parvovirus 4 genotypes 1, 2, and 3). ClustalW-aligned genomes (A) and nonstructural (NS) protein (B) and viral protein (VP) (C) were all trimmed to obtain sequences with similar lengths. All computer analysis was performed by using the neighbor-joining method. Branches corresponding to partitions reproduced in <70% bootstrap replicates are collapsed. The tree is drawn to scale, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown below the branches. F8-year, year of the factor VIII lot; Cl-year, plasma samples and year of collection. Scale bar represents the number of nucleotide (A) or amino acid (B, C) substitutions per site.

showed an alternative coding sequence inside of the VP gene with a recognizable relationship to small alternatively translated proteins (SAT) (*13*). In the porcine PARV4-like viruses, the start codon for the SAT protein was 3 nt downstream relative to the position of SAT-ATG in the human and bovine PARV4 viruses. Although the SAT protein was 67 aa in all the characterized human PARV4 viruses, porcine and bovine PARV-like viruses contained SAT proteins with 84 aa. The amino acid sequences of the SAT proteins were highly conserved in each PARV4 virus group; however, they differed greatly between PARV4 viruses belonging to different host species (Table 2).

Conclusions

Improved virus detection methods have facilitated the discovery of new viruses and have provided insight into the existence of a wide variety of potentially pathogenic strains in biopharmaceutical products. Plasma samples, collected from individual pigs in 2001–2002, and FVIII samples, prepared during 1994–2001, were tested for PARV4-like viruses.

Sequence analysis showed that PARV4-like viruses may have undergone some degree of selective pressure during this time because the genomes sequenced showed a greater variability than the porcine parvovirus NS sequences isolated from the same samples (J. Szelei and P. Tijssen, unpub. data). In the current study, comparison of the genomic and NS protein coding sequences indicated that viruses in the older samples were more closely related to BHoV and PARV4-g2 (Figure 2). Fewer changes were observed in the VP coding sequence (Table 2). Because VPs are responsible for the entry of parvoviruses, they usually adapt to host-specific receptor(s). The presence of PARV4-g2–like isolates in older samples and the omnipresence of PARV4-like viruses in more recent samples suggested that the porcine PARV4-like virus and human PARV4 may have similarly evolved (*8*). These new parvovirus isolates from Great Britain would belong to a different cluster of porcine PARV4-like viruses than the hokoviruses from Hong Kong Special Administrative Region.

Although older isolates shared more identity with BHoV and PARV4-g2, the substantial differences in the DNA sequences of PARV4-like viruses from different species (human, bovine, pig) suggested that they would have diverged a long time ago. This hypothesis was also supported by the sequence stabilization of the SAT proteins, which may play important host-specific roles in the viral exit (*13*). Nevertheless, the existence of a wide variety of different PARV4 strains, most of which result in chronic infections, could provide a basis for an evolutionary jump and recombination and should raise major concerns about the dangers of parenteral transmission.

This research was supported by a grant to P.T. from the Natural Sciences and Engineering Council of Canada.

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Rhabdomyolysis and Pandemic (H1N1) 2009 Pneumonia in Adult

To the Editor: A 56-year-old man came to the emergency department (ED) of Malcolm Grow Medical Center at Andrews Air Force Base in suburban Maryland, USA, just outside Washington, DC. He had a history of several days of cough, fever, and malaise; was a nonsmoker; was overweight (body mass index 28 kg/m^2); and did not have chronic pulmonary disease. Radiographs showed bilateral pulmonary infiltrates, and he was hypoxemic. Two weeks previously, the patient had begun receiving therapy for recurrent multiple myeloma (lenalidomide and high-dose dexamethasone). He was intubated at the time of initial visit to the ED for influenza symptoms, and broad-spectrum antimicrobial drugs were administered (vancomycin 1,000 mg every 12 h, piperacillin-tazobactam 4.5 gm every 6 h, and levofloxacin 750 mg 1×/d). Initial nasopharyngeal wash was negative for influenza A and B antigen by enzyme immunoassay; serum creatine kinase was 271 U/L (reference range 38–174 U/L).

After oxygenation worsened, bronchoalveolar lavage was performed, and drug therapy was broadened to include voriconazole, trimethoprim-sulfamethoxazole, and prednisone. Bronchoalveolar lavage viral culture was positive for influenza A, and real-time reverse transcription–PCR confirmed pandemic (H1N1) 2009 virus infection. Therapy with oseltamivir (75 mg every 12 h) was initiated, and the patient's respiratory status gradually improved. On hospital day 14, total creatine kinase levels were elevated at 4,854 U/L and rose to 76,015 U/L over the next 4 days before decreasing. Urine myoglobin peaked at 286,000 μg/L (reference range 0–28 μg/L). Renal function remained at baseline until the patient was discharged 2 weeks later; measured glomerular filtration rates were >120 mL/min. Hydration with normal saline and supportive care was provided; the patient was extubated and discharged to a rehabilitation hospital on hospital day 19. No medications or other treatments could be implicated as the cause of rhabdomyolysis in this patient.

More commonly reported in children, myositis associated with influenza A and B has been well documented and appears to occur most often during the convalescent phase of illness (*1*). Influenza-associated rhabdomyolysis with myoglobinuria have been shown to complicate 3% of cases of myositis in children, are more likely to be associated with influenza A infection (*1*), and have been associated with renal insufficiency requiring renal replacement therapy (*1*,*2*). The frequency of myositis or rhabdomyolysis among adults with pandemic (H1N1) 2009 infection is unclear, but a recently published case series of 18 severely ill patients in Mexico showed that mild to moderate creatine kinase elevation (1,000–5,000 U/L) occurred in >60% of tested patients (*3*). A report from Australia documented rhabdomyolysis as a complication of pandemic (H1N1) 2009 infection in a 16-year-old-boy (*4*), and, more recently, a case of rhabdomyolysis was reported in a 28-year-old patient (*5*).

Our case demonstrates rhabdomyolysis with myoglobinuria that arose during convalescence from severe pandemic (H1N1) 2009 pneumonia in an immunocompromised adult. It is yet to be determined whether pandemic (H1N1) 2009 virus infection has a higher propensity toward muscular inflammation than do other viral infections or seasonal influenza. Rhabdomyolysis should be considered in the evaluation of muscle symptoms associated with pandemic (H1N1) 2009 virus infection, especially among critically ill patients. When influenza suspicion is high, obtaining bronchoalveolar lavage specimens for viral culture, PCR, and antigen testing should be considered if nasopharyngeal sampling and testing for influenza antigen and viral culture are initially negative.

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DOI: 10.3201/eid1603.091818

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Yersinia pseudotuberculosis and *Y. enterocolitica* Infections, FoodNet, 1996–2007

To the Editor: *Yersinia pseudotuberculosis*, a gram-negative zoonotic bacterial pathogen, causes acute gastroenteritis and mesenteric lymphadenitis, which are often accompanied by fever and abdominal pain. Although *Y. pseudotuberculosis* infections are distributed worldwide, little is known about their incidence and epidemiology in the United States. *Y. pseudotuberculosis* was first reported in the United States in 1938 and has rarely been identified since then (*1*). No outbreaks have been reported, and only 14 cases were documented from 1938 through 1973 (*2*). Although not reportable nationally, yersiniosis is a notifiable disease in all Foodborne Diseases Active Surveillance Network (FoodNet) sites. We describe the *Y. pseudotuberculosis* infections reported through FoodNet surveillance sites and compare these infections with those caused by the more commonly identified *Yersinia* species, *Y. enterocolitica*.

During 1996–2007, FoodNet conducted active surveillance for laboratory-confirmed *Yersinia* spp. infections (excluding *Y. pestis*) in Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, and selected counties in California, Colorado, and New York. All clinical laboratories in these areas were routinely contacted to ascertain cases. Demographic and outcome (e.g., hospitalization and death) information was collected for all cases. On the basis of the source of specimen collection, infections were categorized as invasive (isolated from cerebrospinal fluid, blood, or another normally sterile site) or noninvasive (isolated from urine, stool, or other site). Data were analyzed by using SAS version 9.2 (SAS Institute, Cary, NC, USA). Differences were evaluated by using χ^2 and Fisher exact tests, and medians were compared by using the Wilcoxon rank-sum test. A 2-tailed p value of <0.05 was considered significant.

During 1996–2007, 1,903 *Yersinia* infections were reported in FoodNet sites. Of these, 1,471 (77%) had species information available. Most of the isolates were *Y. enterocolitica* (1,355; 92%); 18 (1%) *Y. pseudotuberculosis* infections were identified. The average annual incidence of *Y. pseudotuberculosis* infections was 0.04 cases per 1,000,000 persons. Most *Y. pseudotuberculosis* cases were reported from the western FoodNet areas of California (5 cases) and Oregon (5 cases).

The median age of persons with *Y. pseudotuberculosis* infection was 47 years (range 16–86 years), and 67% were male (Table). Of the 13 *Y. pseudotuberculosis* cases for which race was reported, 10 (77%) were in whites. Eight (44%) *Y. pseudotuberculosis* cases occurred in the winter months (December–February). Thirteen (72%) persons with *Y. pseudotuberculosis* infection required hospitalization; the median hospital stay was 9 days (range 2–35 days). Two deaths were reported, yielding a case-fatality rate of 11%. Twelve (67%) of the *Y. pseudotuberculosis* isolates were recovered from blood specimens, and only 1 isolate was recovered from stool.

In comparison, the average annual incidence of *Y. enterocolitica* infections in FoodNet was 3.5 cases per 1,000,000 persons, and many of the cases occurred in the southern FoodNet site of Georgia (443 cases, 33%) (Table). Persons with *Y. enterocolitica* infection were significantly younger than those with *Y. pseudotuberculosis* infection (median age 6 years, p = 0.0002), and unlike *Y. pseudotuberculosis* infections, *Y. enterocolitica* infections were evenly distributed among male and female patients and among whites and blacks. Compared with those with *Y. enterocolitica* infection, persons with *Y. pseudotuberculosis* infection were more likely to be hospitalized (p = 0.0003), have longer hospital stays (p = 0.0118), die (p = 0.0248), and have an isolate recovered from an invasive site (p<0.0001).

Most of the *Y. pseudotuberculosis* infections reported in FoodNet sites appeared to be severe and invasive. The rarity of diagnosed *Y. pseudotuberculosis* infections is consistent with earlier reports from North America (*3*,*4*), but

Table. Comparison of *Yersinia pseudotuberculosis* and *Y. enterocolitica* infections, FoodNet, 1996–2007*

Characteristic	*Y. pseudotuberculosis*	*Y. enterocolitica*	p value
No. infections	18	1,355	
Annual average incidence† (range)	0.04 (0.00–0.10)	3.45 (0.77–7.87)	
Median patient age, y (range)	47 (16–86)	6 (0–94)	<0.0001
Male sex, no. (%) patients	12 (67)	672 (50)	0.1638
White race, no. (%) patients	10 (56)	480 (35)	0.0115
Western region of USA (CA, OR), no. (%)	10 (56)	308 (22)	0.0024
Winter season, no. (%)	8 (44)	536 (40)	0.8091
Invasive specimen collection site, no. (%)	12 (67)	106 (8)	<0.0001
Hospitalized, no. (%) patients	13 (72)	411 (30)	0.0003
Median hospitalization, d (range)	9 (2–35)	4 (0–107)	0.0118
Died, no. (%) patients	2 (11)	15 (1)	0.0248

*CA, California; OR, Oregon.
†Cases per 1,000,000 persons.

this rarity remains unexplained. This rarity contrasts with the observation that cases and outbreaks are more common in other parts of the developed world, particularly in northern climes (*1*,*5*,*6*–*8*). The recent appearance of epizootic *Y. pseudotuberculosis* in farmed deer in the southern United States suggests that this could change (*9*).

The high proportion of *Y. pseudotuberculosis* cases that were diagnosed by blood culture suggests that less invasive *Y. pseudotuberculosis* infections are underrecognized in the United States. Diagnosis of *Yersinia* infections is difficult without specific culture, *Yersinia* is not routinely tested for in the United States, and isolation of the organism by culture may be difficult with standard media (*2*,*10*). Clinical diagnosis of *Y. pseudotuberculosis* infections can be challenging because physicians are not aware that *Y. pseudotuberculosis* is a potential cause of gastroenteritis (*10*). In the syndrome of pseudoappendicitis, the distinctive findings found by surgical exploration of severe mesenteric lymphadenitis can be suggestive, but diagnosis would require confirmation by culture of nodes or feces (*2*,*3*).

Unless the physician is both aware of *Y. pseudotuberculosis* as a cause of gastroenteritis and knows which diagnostic test to order, *Y. pseudotuberculosis* infections will go undiagnosed. Clinicians should consider *Y. pseudotuberculosis* as a cause of gastroenteritis and pseudoappendicitis and request appropriate microbiologic testing for patients with suspected cases. If more cases are identified in the United States, another investigation of *Y. pseudotuberculosis* might clarify the epidemiology of this infection.

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DOI: 10.3201/eid1603.091106

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Measles Outbreak, the Netherlands, 2008

To the Editor: From June 1 through October 16, 2008, an outbreak of 99 reported measles cases occurred in the Netherlands (*1*). This outbreak was the largest measles outbreak in the Netherlands since 1999–2000, when ≥3,200 cases, including 3 deaths, were reported (*2*).

In the Netherlands, clinical symptoms compatible with measles in a person with laboratory-confirmed measles virus infection or an epidemiologic link to a laboratory-confirmed case are notifiable (i.e., must be reported to public health authorities). The National Measles Reference Laboratory conducts genotyping and submits sequences to the World Health Organization European Region Measles Nucleotide Surveillance database (www.hpa-bioinformatics.org.uk/Measles/Public/Web_Front/main.php).

Of the 99 measles cases reported in the 2008 outbreak, 40 were laboratory confirmed and 59 were notified based on an epidemiologic link. The first case-patient in the outbreak was a 6-year-old unvaccinated resident of The Hague who had not been abroad in the month before onset of illness.

The source of her infection was unknown. She attended a school based on anthroposophic principles; the school had an estimated measles-mumps-rubella (MMR) vaccination coverage of 80% (M. Monné-van Wirdum, pers. comm.). Subsequently, 52 additional cases were reported from this and from another anthroposophic school in The Hague (cluster 1; online Appendix Figure, www.cdc.gov/EID/content/16/3/567-appF.htm). Two months after the first case, 22 additional cases were reported associated with an anthroposophic summer camp in the east of the Netherlands (cluster 2; online Appendix Figure). Five additional cases had an epidemiologic link with an anthroposophic summer camp in France (cluster 3, 2 cases; online Appendix Figure) and Switzerland (cluster 4, 3 cases; online Appendix Figure). No known measles patients in Switzerland were linked to this cluster (J. Richard, pers. comm.). Subsequently, 12 cases were reported that were associated with 2 daycare centers in the city of Utrecht (cluster 5 and 6), both linked to an anthroposophic community. From all 6 clusters and from 2 of the 7 cases with an unknown source, indistinguishable measles viruses (genotype D8, 22 cases) were identified. Given the low prevalence of this strain in Europe (J. Kremer, pers. comm.), we concluded that virus transmission occurred between all 6 clusters. The first cluster was not epidemiologically linked to any of the recent outbreaks in anthroposophic groups in Europe (*3*).

No case had an epidemiologic link to more than 1 cluster, suggesting the 6 cases introducing measles into these clusters were unreported. When the 7 cases with an unknown source were considered, this finding suggests that at least 13 cases were not reported (maximum reporting completeness 88%). However, transmission through patients with subclinical cases may also have played a role (*4*).

There were no deaths. Four case-patients (4%) were admitted to hospitals. The median age was 9 years (range 8 months–48 years). Of the 98 case-patients with information on vaccination status, 91 (93%) had been unvaccinated, 6 (6%) had had 1 dose, none (0%) had had 2 doses, and 1 (1%) had had 3 doses before onset of illness. One of the 6 case-patients, vaccinated only once, had received her MMR vaccine only 11 days before the date of onset of illness and is hence not considered a vaccine failure. Of all 99 case-patients, 91% had been eligible for ≥1 MMR vaccination according to the vaccination schedule in the Netherlands. Of these cases, available information for 84 case-patients indicated 48% (40 persons) were reported to be unvaccinated because of their anthroposophic beliefs, 49% (41 persons) because of a critical attitude towards vaccination, and 4% (3 persons) for other reasons.

Outbreak control plans in the Netherlands focus on protecting the population by adjusting the vaccination schedule during a nationwide outbreak (*5*). Studies are ongoing into knowledge and attitudes toward vaccination in communities with low vaccination coverage, aiming to identify opportunities to improve coverage.

The outbreak remained largely restricted to persons with philosophical objections to MMR vaccination, which suggests that there are sufficient levels of herd immunity in the general population. Remarkably, no cases were reported from the Dutch Orthodox Reformed Church community, despite the low vaccine coverage in this group. This finding suggests that orthodox reformed and anthroposophic population subgroups have little direct contact, consistent with previous observations (*6*).

Measles vaccination was introduced in the Netherlands in 1976. The single-dose regimen was in 1987 replaced by a 2-dose regimen of MMR vaccine; the first dose at 14 months and the second at 9 years. The vaccination coverage for ≥1 MMR dose has been >95% from birth cohort 1986 onward (*7*). During 2002–2007, the incidence of measles notifications in the Netherlands was below the World Health Organization regional threshold for elimination (1/1 million population/year) (*8*). Nevertheless, this outbreak demonstrates the continued risk for measles transmission in the Netherlands. This suggests that indicators based merely on incidence and national vaccination coverage are of limited usefulness for certification of measles elimination. Data on measles seroprevalence and mixing patterns that will soon be available from the second national seroprevalence study will provide more insight into the dynamics of measles transmission in a population with pockets of low vaccination coverage. These data will also help assess progress toward measles elimination from the Netherlands.

Acknowledgments

We thank the staff of Municipal Health Authorities and Microbiological Laboratories and Leslie Isken for their support in obtaining information on the cases reported.

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DOI: 10.3201/eid1603.090114

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Neurologic Manifestations of Pandemic (H1N1) 2009 Virus Infection

To the Editor: In April 2009, the outbreak of influenza A pandemic (H1N1) 2009 virus was reported. Subsequently, the disease spread throughout the world, and the pandemic alert level was raised to level 6 in June by the World Health Organization. Pandemic (H1N1) 2009 virus infection spread to Thailand and is now found throughout Thailand. Similar to the effects of other viruses, pandemic (H1N1) 2009 virus may cause neurologic complications. Associated neurologic symptoms were first reported from Dallas, Texas, USA: 4 children experienced unexplained seizures or had an alteration of consciousness level that was associated with this virus (*1*). We report an adult patient with pandemic (H1N1) 2009 infection who had neurologic complications.

A 34-year-old man, previously healthy, was admitted to Chaiyaphum Hospital in Chaiyaphum, Thailand, on August 24, 2009, with influenza-like symptoms. Two days after admission, progressive quadriparesis with bilateral, symmetric paresthesia (glove-and-stocking pattern), and areflexia developed. His motor weakness (grades III/V) began in both legs and then involved both arms and hands. Other neurologic examinations showed limitation of extraocular movement in all directions, normal pupil size and light reflex, and facial diplegia. A lumbar puncture was performed, and cerebrospinal fluid (CSF) contained neither leukocytes nor erythrocytes, with a protein level of 19.5 mg/dL.

On day 3 after the patient's admission, acute respiratory failure developed. A nasopharyngeal aspirate specimen was positive for pandemic (H1N1) 2009 virus by PCR. The patient received oseltamivir, zanamivir, and ventilator support. His chest radiograph showed diffuse alveolar infiltration. On day 10, his motor weakness worsened to grade 0, and his consciousness level was diminished to a drowsy state.

A computed tomography scan of the brain showed diffuse white matter lesions (Figure). Repeated lumbar punctures continued to show CSF findings within the reference range. An electrophysiologic study, electromyogram, and nerve conduction study showed polyneuropathy, axonopathy type. Guillain-Barré syndrome was suspected, and intravenous immunoglobulin was given for 5 days. Tests for GQ1b and GM1 antibodies were carried out at Oxford University; results were negative.

Other laboratory tests showed mild transaminitis and negative results for syphilis testing and for serologic tests for HIV, hepatitis B virus, hepatitis C virus, Japanese encephalitis virus, herpes simplex virus, and *Mycoplasma pneumoniae*. A CSF antigen test was negative, and CSF culture was negative for bacteria. Meropenem was given to treat ventilator-associated pneumonia, which was caused by β-lactam–resistant *Klebsiella pneumoniae*. After a month of treatment, the patient regained consciousness, his motor strength improved considerably, and he was able to be gradually removed from the ventilator. After 3 months, he was discharged with self-assisted status.

Our report shows neurologic manifestations associated with pandemic (H1N1) 2009 virus infection in an adult. The manifestation of progressive quadriplegia with diffuse sensory loss is compatible with a polyneuropathy. The neurologic signs developed 2 days after the respiratory tract signs.

Although a diagnosis of Guillain-Barré syndrome was considered initially, according to the National Institute of Neurologic Disorders and Stroke criteria (*2*), some clinical features did not support this diagnosis.

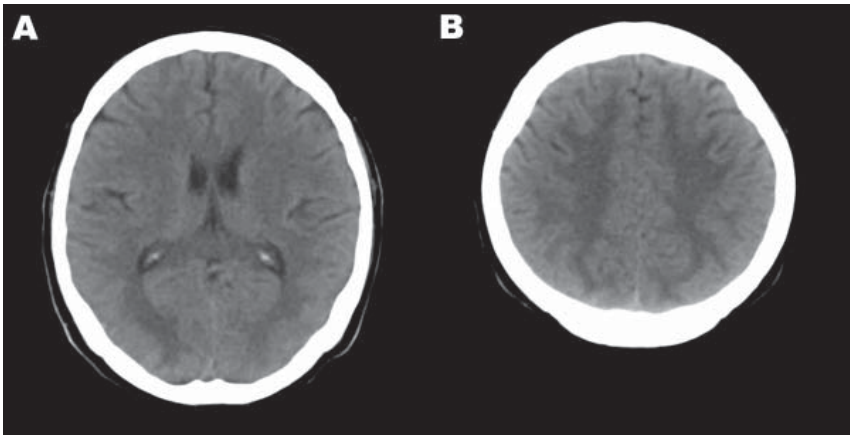

Figure. Computed tomography images of the brain of an adult patient with pandemic (H1N1) 2009 virus infection and neurologic signs. A noncontrast study showed hypodense lesions in both occipital lobes (A) and in both upper parietal lobes (B).

These included the lack of CSF albuminocytologic dissociation, the fact that the clinical signs occurred during the outbreak of pandemic (H1N1) 2009 virus infection rather than after it, and the fact that antibodies were not found in gangliosides. CSF albuminocytologic dissociation and serum ganglioside antibodies may be found in 85%–90% of Guillain-Barré syndrome patients (*2*).

Alternatively, the patient might have had central nervous system complication from pandemic (H1N1) 2009 virus infection. Acute disseminated encephalomyelitis is a condition that might occur within 30 days after an infectious process (*3*). It can lead to quadriplegia and diffuse white matter lesions. The clinical feature that makes acute disseminated encephalomyelitis less likely in this patient was the CSF findings in the reference range. In summary, however, we believe that pandemic (H1N1) 2009 virus infection can cause neurologic complications affecting both the peripheral and central nervous systems in adult patients.

This work was supported by the Office of the Higher Education Commission and Khon Kaen University, Thailand.

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DOI: 10.3201/eid1603.091699

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Rickettsia felis, West Indies

To the Editor: A spay–neuter (sterilization) program for feral cats from Basseterre, the capital of the Caribbean Island St. Kitts, found that most (45/58; 66%) cats had antibodies to spotted fever group rickettsiae (SFGR). The antibodies were detected with *Rickettsia rickettsii* antigen in a standard microimmunofluorescence assay (*1*). Titers for 13 (20%) cats were ≥320.

Most SFGR are transmitted by ticks, but because of their grooming habits, cats seldom have many ticks (*2*), and we did not find any ticks on the cats we saw through the program. We did, however, commonly find cat fleas, *Ctenocephalides felis*, which are the main vector of *R. felis*, a recently described member of the SFGR. *R. felis* seems to be apathogenic in cats (*3*) but is the agent of flea-borne spotted fever in humans (*4*). Although *R. felis* has been reported from North and South America, Europe, Africa, the Middle-East, and Oceania (*4*), its presence in the Caribbean islands has not been established. To provide this information we tested DNA extracted with the QIAamp DNA Mini-Kit (QIAGEN, Valencia, CA, USA) from *C. felis* fleas preserved in 70% ethanol.

Of 57 (19%) *C. felis* fleas from St. Kitts, 11 were positive for *R. felis* DNA when tested by PCR using primers targeting SFGR *omp*A (*5*) or TaqMan assay using primers targeting *glt*A and a probe specific for the organism (*6*,*7*). For a negative control we used distilled water; for a positive control we used DNA from *R. montanensis* cultures or recombinant control plasmids constructed by amplifying target fragments from *R. typhi* strain Wilmington and *R. felis* strain LSU (*7*). The sequences of the *omp*A and *glt*A amplicons obtained had 100% nucleotide sequence similarity with homologous fragments of the type reference isolate *R. felis* URRxCal2. We used the Na-

tional Center for Biotechnology Information basic local alignment sequence tool, BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

To determine whether *R. felis* occurs on another Caribbean island, we tested 32 *C. felis* fleas from Dominica and found 1 (3%) to be positive by PCR when primers targeting *omp*A were used. The sequence obtained was also identical to that of *R. felis* URRxCal2.

Our study provides further evidence that cats can be sentinels for the presence of rickettsiae (*1*). However, although rickettsemia can develop in cats experimentally infected with *R. felis* (*3*), no compelling evidence shows that cats help maintain the organism or transmit it to humans (*8*,*9*). Rather, it appears that *C. felis* fleas, which are also commonly found on dogs and to a lesser extent other mammals, are the major reservoir hosts and vectors of infection, although the exact mechanisms are unknown (*10*). Our study also expands the known distribution of *R. felis* and should alert healthcare workers who see residents of or vacationers from the Caribbean islands of the possibility of flea-borne spotted fever in their patients.

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DOI: 10.3201/eid1603.091431

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Rickettsia africae, Western Africa

To the Editor: *Rickettsia africae*, the causative agent of African tick-bite fever, is transmitted by *Amblyomma hebraeum* and *A. variegatum* ticks (*1*,*2*). These ticks are common in western, central, and southern Africa. Adults rarely feed on humans, although nymphs attach more frequently and larvae are sometimes serious pests (abundant and aggressive) (*3*).

African tick-bite fever is a neglected disease that has been mainly detected in tourists who were bitten by a tick while traveling in disease-endemic areas (*2*). A recent worldwide report showed rickettsial infection incidence to be 5.6% in a group of travelers in whom acute febrile infection developed after they returned from sub-Saharan Africa. African tick-bite fever is the second most frequently identified cause for systemic febrile illness among travelers, following malaria (*4*). Seroprevalence for spotted fever group rickettsiae is high in the Sahel regions of Africa (*5*), although there may be different emergent and classic rickettsioses in Africa.

R. africae has been detected by PCR in many African countries, including Niger, Mali, Burundi, and Sudan (*6*), and in most countries of equatorial and southern Africa (Figure). Most strains and cases have been found in South Africa (*2*). *R. africae* and African tick-bite fever have not previously been reported in Senegal, and few positive human serum samples have been documented in western Africa. *A. variegatum*, the main vector of *R. africae*, was introduced by cattle into Guadeloupe, West Indies, from Senegal in the early 1800s. Spotted fever caused by *R. africae* has become endemic there in the past 30 years (*7*). In addition to *R. africae*, *A. variegatum* ticks may transmit other human and animal pathogens, including Crimean-Congo hemorrhagic fever virus, Dugbe virus, Thogoto virus,

Bhanja virus, *Ehrlichia ruminantium*, *Theileria* spp., *Anaplasma* spp., and *Dermatophilus congolensis* (*3*,*6*).

From November through December 2008, ticks were collected from domestic animals (cattle, goats, sheep, dogs, horses, donkeys) in the Sine-Saloum region of Senegal (villages Dielmo, Ndiop, Medina, and Passi). Among the collected ticks, 8 fully engorged nymphs were kept alive in flasks at 90%–95% relative humidity. Other ticks were stored in 70% ethanol. Flagging at ground level was used to collect ticks from pastures. Species were identified according to standard taxonomic keys for adult ticks. Nymphs were allowed to molt before identification and subsequent bacterial culture. Rickettsial DNA in other ticks was detected by semiquantitative PCR with *Rickettsia*-specific primers (*8*). All positive samples were subjected to PCR by using primers designed for the *gltA* and *ompA* genes (*6*). Three rickettsial spacers were chosen for typing: *dksA-xerC*, *rpmE-tRNAmet*, and *mppA-purC* (*9*).

Tick larvae were the only stage collected by flagging at ground level. Flagging for 30 minutes collected 495 larvae near the village of Passi and 325 in Dielmo. The larvae were aggressive, and several attached onto the collector's ankles despite preventive measures. All larvae were morphologically identified as *Amblyomma* spp. Amplification and sequencing of the portion of mitochondrial cytochrome oxidase I gene of 3 adult *A. variegatum* ticks, 2 individual larvae, and 1 pool of 10 larvae detected a 659-bp sequence 100% identical among all larvae and adults and corresponding to cytochrome oxidase I of other ticks. The sequence is deposited in GenBank, accession no. GU062743.

Adult ticks (n = 492) were collected from domestic animals; 85 (17.3%) were *A. variegatum,* and 74 (87.1%) were positive for rickettsial genes according to real-time PCR. No associations between animal host, place of collection, and presence of *R. africae* were found (data not shown). During the subsequent amplification and sequencing of the 632-bp fragment of the *ompA* gene, all amplicons were found to be 100% identical to the *ompA* sequence of *R. africae* published in GenBank (CP001612.1).

Molted nymphs were the source of 3 strains of *R. africae*. Although dogs are rarely reported to be hosts of *A. variegatum* (*3*), a dog was the host of the tick that carried the first isolated strain. A 1,290-bp fragment of the rickettsial *gltA* gene and a 632-bp fragment of the *ompA* gene from all 3 strains were identical to the published sequence of the *R. africae* genome (CP001612.1). Multispacer typing showed that all 3 *R. africae* strains exhibited a genotype identical to that of all previously genotyped *R. africae* strains (genotype 38). To the best of our knowledge, this is the northernmost reported isolation of this pathogen in western Africa.

Taking into consideration data described in previous studies and the results of our work, we conclude that *A. variegatum* is an aggressive and abundant species of tick. The reported transovarial transmission rate of 100% for *R. africae* (*10*), the abundance of ticks, and the high percentage of ticks that are infected (*3*) increase the probability that humans will be bitten by infected ticks. *R. africae* is present in Senegal, and human infections (in tourists and indigenous populations) may be as common there as in southern Africa, but better availability of diagnostic assays is needed. Surveys of the distribution of vector ticks and rickettsiae should be performed, and target groups should be screened.

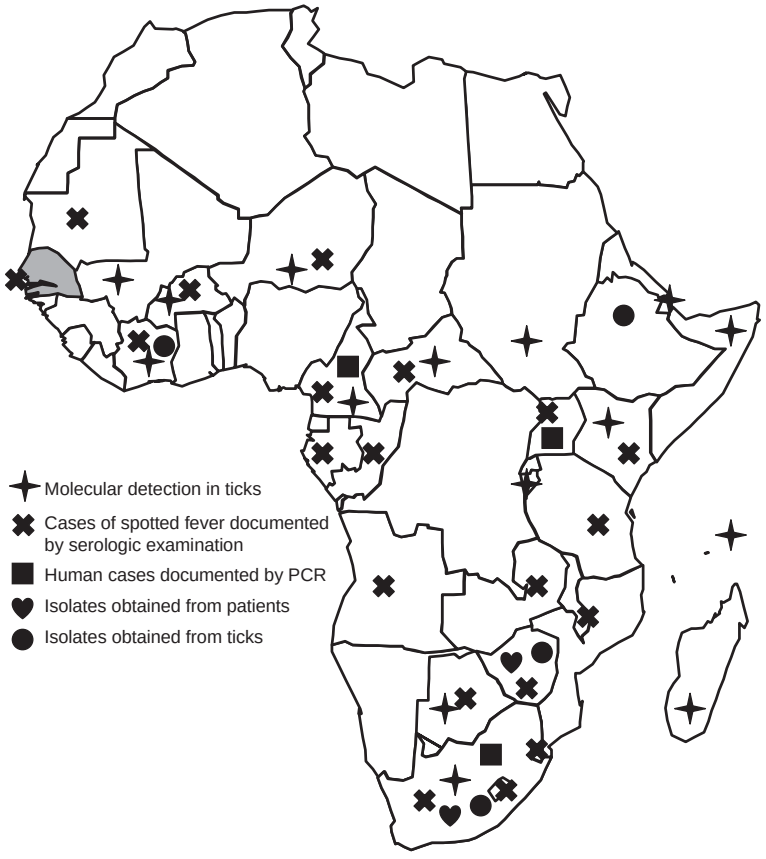

Figure. Distribution of *Rickettsia africae* in the African continent and serologic evidence of spotted fevers in humans. Gray shading indicates location of Senegal.

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DOI: 10.3201/eid1603.090346

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Transmission of West Nile Virus during Horse Autopsy

To the Editor: West Nile virus (WNV) circulates mainly in birds and ornithophilic mosquitoes. Humans and horses are considered incidental, dead-end hosts (*1*). Fever, rash, arthralgia, and myalgia develop in ≈20% of cases in humans; severe neurologic disease may develop in <1% (*1*). In horses, 20% of infections result in clinical disease, of which ≈90% involve neurologic disease with ataxia, weakness, recumbency, muscle fasciculation, and high death rates (30%) (*2*).

Genetic variants of WNV include lineage 1 found in the Northern Hemisphere and Australia; lineage 2 found mainly in southern Africa and Madagascar (*3*); lineages 3 and 4 found in central and eastern Europe (*4*); and lineage 5 found in India (*5*). Differences in neuroninvasiveness and pathogenic potential are functions of individual genotypes, not lineage (*3*,*6*–*8*).

We recently reported WNV lineage 2 in several cases of neurologic disease in horses in South Africa (most cases were fatal) (*7*). We report a case of zoonotic transmission to a veterinary student during the autopsy of a horse. The study was reviewed and approved by the Ethics Committee of the University of Pretoria, and informed consent was provided by the veterinary student.

On April 9, 2008, a 4-month-old Welsh pony from Gauteng in South Africa had fever, Schiff-Sherrington signs, and a leukocyte count of 32×10^9 cells/L. He was treated with dimethyl sulfoxide, dexamethasone, and chloramphenicol and responded well. He was able to stand with help, and did not show neurologic signs at this stage. On May 9, he was sent home and was able to walk with support. On May 12, he had a relapse with neurologic deterioration and rectal prolapse, and was treated with antiinflammatory agents. Symptoms worsened and he was humanely killed on May 15 by using ketamine and $MgSO_4$. The carcass was sent to the Faculty of Veterinary Sciences, University of Pretoria, for autopsy because of unusual neurologic signs in the pony. Autopsy was performed by a veterinary pathologist and 2 students on May 16, 2008.

Macroscopic findings included moderate intermuscular, fascicular, perineural edema, severe diffuse pulmonary edema, mild hydropericardium, and rectal prolapse resulting in marked submucosal edema and mucosal hyperamia, i.e., traumatic proctitis. The spinal cord up to C1 showed marked Wallerian degeneration of the peripheral white matter from the median fissure, which extended along the ventral funiculus up to the most dorsal section of the lateral funiculus. Changes were characterized by white matter spongiosis with numerous digestion chambers containing phagocytosing myelinophages and scattered interstitial gemistocytes. No inflammatory reaction was detected. We also observed septal edema and moderate multifocal perivascular and peribronchiolar lymphocytic infiltration with occasional apoptosis in the lungs.

The brain, which was removed by 1 of the students, was sent to the Department of Medical Virology, University of Pretoria for WNV reverse transcription–PCR (RT-PCR). The lungs were sent to Onderstepoort Veterinary Institute for African horse sickness RT-PCR. WNV-specific real-time RT-PCR showed positive results. DNA sequencing and phylogenetic analysis identified WNV lineage 2 in several sections of the brain (HS23/2008, GenBank accession no. FJ464376). Results of African horse sickness RT-PCR on lung tissue specimens were inconclusive and could not be confirmed by culture.

On May 22, six days after the autopsy on the horse, fever, malaise, myalgia, stiff neck, and severe headache developed in the veterinary student who had handled the horse brain. A rash appeared 2 days later. Symptoms persisted for ≈10 days. The patient was treated symptomatically by an infectious disease specialist and prescribed bed rest. Because cases of Rift Valley fever were recently reported in veterinarians in South Africa, serum was sent to the National Institute for Communicable Diseases, where a virus isolate was obtained in suckling mice and identified as WNV by RT-PCR.

After diagnosis of WNV infection in the pony, RNA extracted from the original human serum and from the suckling mouse isolate was sent to the Department of Medical Virology, University of Pretoria, for DNA sequencing and phylogenetic analysis of the virus. Comparison of part of the nonstructural protein 5 gene identified identical sequences from the student's serum, the virus isolate, and the pony's brain. All sequences clustered with lineage 2 WNV and were closely related to isolates obtained from horses diagnosed with fatal WNV encephalitis in South Africa in 2008 (*7*) (Figure).

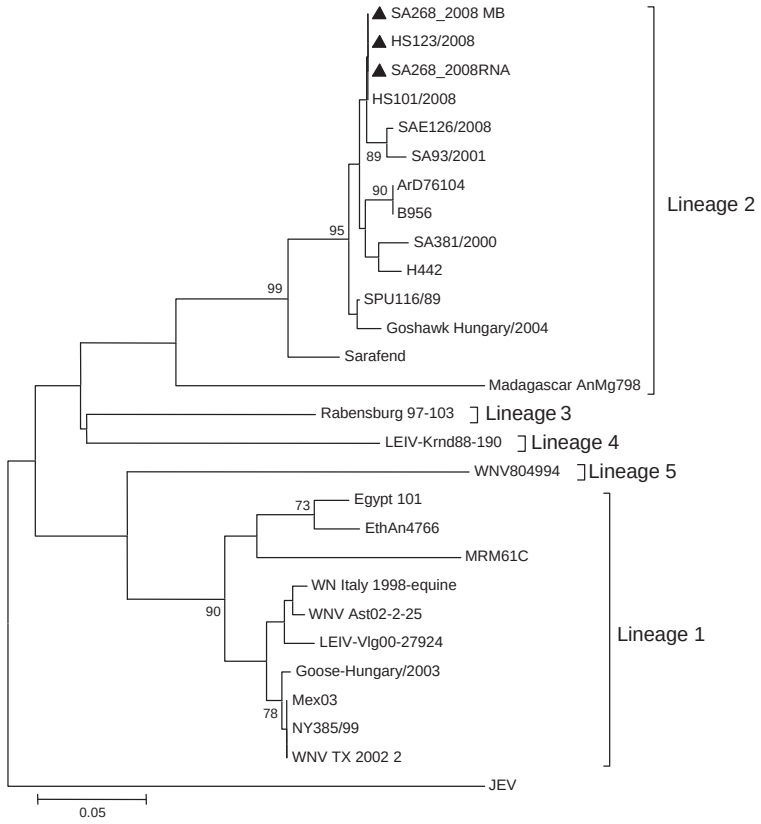

Figure. Phylogenetic comparison of West Nile virus (WNV) nonstructural protein 5 partial gene fragment identified in a veterinary student's serum and in the virus isolate obtained from mouse brain and the horse's brain after autopsy (triangles) relative to other WNV strains from South Africa and elsewhere. The neighbor-joining tree was compiled by using MEGA version 4 software (www.megasoftware.net/under) and 1,000 bootstrap replicates by using the maximum composite likelihood algorithm. Genetic lineages are indicated on the right as described (*3*–*7*). Scale bar indicates nucleotide substitutions per site. JEV, Japanese encephalitis virus (included as outgroup).

Human infections with WNV have been described after bird autopsies and needle stick injury in laboratory workers (*9*,*10*). The case acquired by our patient suggests a zoonotic risk exists for infection with WNV during autopsy of horses that died from neurologic disease. Although humans and horses are considered to have low-grade viremia, virus levels may be higher in nerve tissue.

The patient wore latex gloves, his only protection during the autopsy, and had removed the spinal cord and brain. No protective inhalation or eye equipment was worn. No autopsy assistants or other students who worked with or were near the carcass became sick or seroconverted. The most likely route of infection may have involved exposure of mucous membranes to droplets. After the incident, biosafety measures were improved and included wearing of masks and eye protection gear during autopsies at the facility.

Acknowledgments

We thank Janice Croft and Busi Mogodi for technical assistance.

This study was supported by a grant from the National Research Foundation of South Africa.

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DOI: 10.3201/eid1603.091042

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Breeding Sites of Bluetongue Virus Vectors, Belgium

To the Editor: Bluetongue (BT) is an emerging disease of ruminants in northern Europe (*1*,*2*). This disease was reported in August 2006 in the Netherlands and a few days later in Belgium. In 2006, animals in the Netherlands, Belgium, and Germany were affected. In contrast to 2006, when BT virus (BTV) was identified in ≈2,000 enclosures on farms, BTV was identified in >40,000 farm buildings containing ruminants in 2007; many infected animals had severe disease. In addition, the virus expanded its range to include large areas of France, Denmark, the United Kingdom, Switzerland, and the Czech Republic (*2*).

In 2008, BTV serotype 8 (BTV-8) continued its spread across Europe and showed virulence in France where 26,925 BTV-8 outbreaks were reported (*3*). This observation indicates possible overwintering of the vector from year to year. However, the mechanism of overwintering is not clear. The biting midges responsible for transmission of BTV in northern Europe belong to the genus *Culicoides*, but only few species are vectors of this virus (*2*).

During the winter of 2006–2007, Losson et al. (*1*) monitored the presence of biting midges inside farm buildings. Zimmer et al. (*4*) observed potential vectors of BTV inside a sheepfold during the winter of 2007–2008 and in farm buildings in 2008. These authors suggested that *Culicoides* spp. may be more abundant indoors than outdoors when animals are kept in these buildings. Breeding sites of bluetongue vector species have been found near farms (silage residues) (*5*) and in neighboring meadows (overwintering cattle dung and silt along a pond) (*5*,*6*) but not inside sheds.

We conducted a study on 5 cattle farms in Belgium during February–October 2008. Three samplings were performed: the first in late February, the second in mid-June, and the third in late October. Soil samples (15 biotopes) were collected inside cowsheds. These samples were incubated at 24°C to enable adult midges to emerge. All *Culicoides* specimens were identified by sex and to the species level by using the morphologic key of Delécolle (*7*).

Among 15 soil biotopes obtained from farm buildings, only 1 showed the emergence of adult *Culicoides* biting midges. At a cattle farm in Spy (50°28′31″N, 4°40′39″E), we found that dried dung adhering to walls inside animal enclosures and used animal litter was a breeding site for the *C. obsoletus/scoticus* complex (Table). Only 25% of emerging *Culicoides* midges were females.

We observed that *C. obsoletus/scoticus* complex midges are more prevalent in soil samples with a high carbon:nitrogen (C:N) index; this index indicates the amount of organic matter in soil. C:N indices between 15 and 30 support production of humus and ensure good microbial growth. In addition, larvae of *Culicoides* spp. feed on organic material and microorganisms in soil (*8*).

Our observations suggest that biting midges can complete their life cycle in animal enclosures. This finding is consistent with the high capture

Table. *Culicoides* species obtained from dried dung samples inside a cowshed, Spy, Belgium, 2008

Sampling period	Carbon: nitrogen index	No. *Culicoides* specimens	*Culicoides* species	
			C. obsoletus males	*C. obsoletus/scoticus* females
Late February	19.5	53	40	13
Mid-June	12.8	13	10	3
Late October	12.5	3	2	1

rates of nulliparous (empty and unpigmented abdomens) (*9*) adult midges observed when suction light traps (Onderstepoort Veterinary Institute, Onderstepoort, South Africa) were used on cattle farms during April–May 2007 (*4*).

We identified a breeding site for the primary BTV vector in a cowshed in northern Europe (*10*). Vectors feed on blood, overwinter inside cowsheds (*1*), lay eggs, and larvae develop under such conditions. These observations could explain the persistence of BTV from year to year despite fairly harsh winters.

Hygienic measures on farms could reduce midge populations and improve efficacy of vaccination campaigns against BT in Europe. We strongly recommend that such integrated control strategies be evaluated. Removal of residual animal feed and feces on farms and of material from silage structures and sheds, particularly deposits of manure adhering to walls of sheds and used litter, are simple and inexpensive measures that should be implemented. However, their success will depend on active participation by farmers.

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DOI: 10.3201/eid1603.091311

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Two Lineages of Dengue Virus Type 2, Brazil

To the Editor: Dengue viruses (DENVs) belong to the genus *Flavivirus* (family *Flaviviridae*) and exist as 4 antigenic types, serotypes 1–4, each with well-defined genotypes. Dengue virus is associated with clinical manifestations that range from asymptomatic infections and relatively mild disease (classic dengue fever) to more severe forms of dengue hemorrhagic fever and dengue shock syndrome. Dengue has become one of the most serious vector-borne diseases in humans. The World Health Organization estimates that 2.5 billion persons live in dengue-endemic areas and >50 million are infected annually (*1*).

In 1986, dengue virus type 1 (DENV-1) caused an outbreak in the state of Rio de Janeiro and has since become a public health concern and threat in Brazil. (*2*). In 1990, DENV-2 was reported in the state of Rio de Janeiro, where the first severe forms of dengue hemorrhagic fever and fatal cases of dengue shock syndrome were documented. The disease gradually spread to other regions of the country (*3*). In 2002, DENV-3 caused the most severe dengue outbreak in the country and sporadic outbreaks continued to be documented through 2005 (*4*).

Since 1990, two additional epidemics caused by DENV-2 have occurred (1998 and 2007–2008) in Brazil. A severe DENV-2 epidemic in the state of Rio de Janeiro began in 2007 and continued in 2008; a total of 255,818 cases and 252 deaths were reported (*5*). This epidemic prompted us to investigate the genetic relatedness of DENV-2 for all of these epidemics.

DENV-2 isolates from these epidemic periods were subjected to sequencing and comparison. Gross sequences of DENV-2 isolates from all epidemic periods grouped with sequences from DENV-2 American/

Asian genotype; this finding was expected because this genotype is circulating in the Americas (*6*,*7*). Sequences of DENV-2 isolates from the 1998 epidemic grouped with sequences of DENV-2 isolates from the 1990 epidemic (data not shown) suggesting that viruses circulating during these 2 epidemic periods belong to the same lineage of the DENV-2 strain originally found in the state of Rio de Janeiro. However, sequences of DENV-2 isolates from 2007/2008 epidemics grouped separately and distinctly from the 1990 and 1998 DENV-2 isolates and represented a monophyletic group in the phylogenetic tree with bootstraps of 98% (Figure). This result shows a temporal circulation of genetically different viruses in Rio de Janeiro that could be a result of local evolution of DENV-2 since its introduction in 1990, or even an introduction of a new lineage of DENV-2 in the region.

A study conducted by Aquino et al. (*7*) showed that Paraguayan DENV-2 strains could be grouped as 2 distinct variants within the American/Asian genotype, thus further supporting that the introduction of new DENV-2 variants may likely associate with the shift of dominant serotypes from DENV-3 to DENV-2 in 2005 and might have caused an outbreak of DENV-2. Our results are consistent with this scenario because was a shift of a dominant serotype from DENV-3 to DENV-2 that was observed in 2008 in Rio de Janeiro. However, other factors, such as immunity level to DENV-3 and DENV-2, could explain the shift of dominant serotype besides the circulation of a new viral variant.

Because the dengue outbreaks of 2007 and 2008 were the most severe of the dengue infections in Brazil in terms of number of cases and deaths, this genetically distinct DENV-2 could have contributed to this pathogenic profile. Additionally, these samples came from disperse locations in Rio de Janeiro and we do not believe that there is a clustering issue in our sampling. However, again, other factors must be considered as contributors to this scenario because of the intrinsic properties of this distinct virus, host susceptibility, and secondary cases of infection.

In addition, detailed examination of amino acid sequences of Brazilian DENV-2 strains isolated in 1998 and

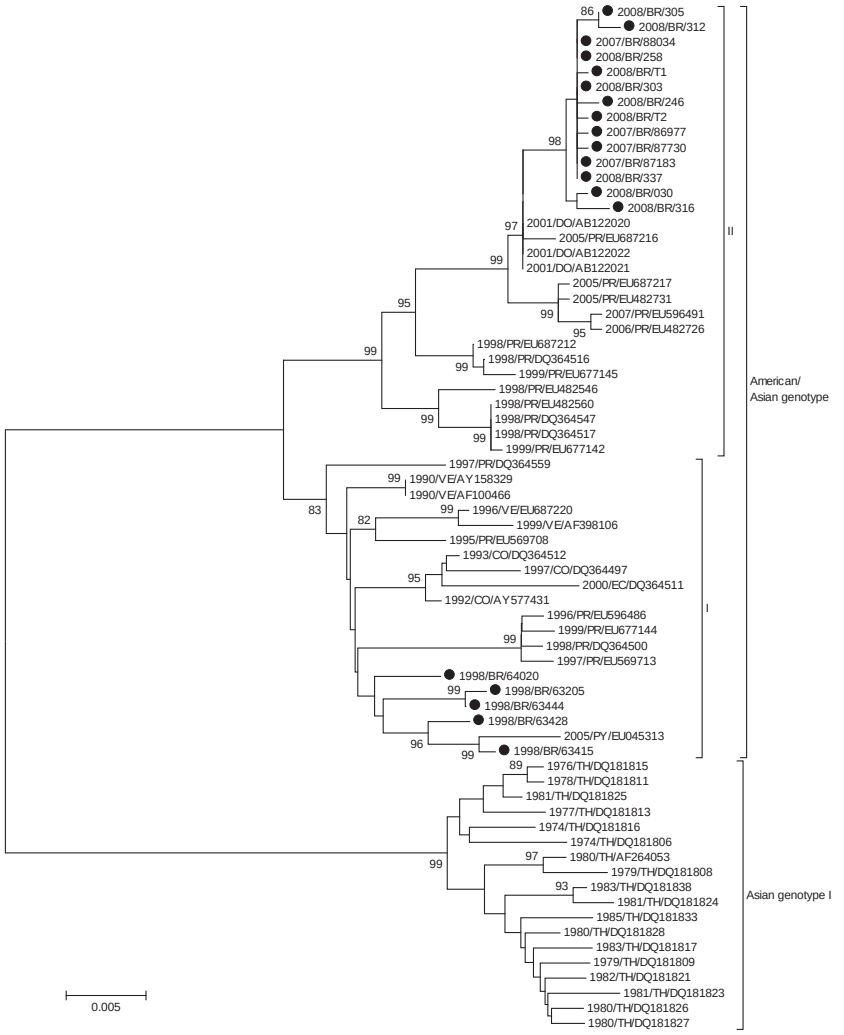

Figure. Neighbor-joining phylogenetic tree of 68 complete envelope (E) gene sequences of dengue virus type 2 (DENV-2). Only bootstrap values >80% are shown. DENV-2 sequences obtained from 21 patients infected during the 1990, 1998, and 2007–2008 epidemics were isolated from acute-phase cases. Sequences of the E gene were compared with DENV-2 sequences of American/Asian genotype deposited in GenBank (www.ncbi.nlm.nih.gov). Strains of Asian genotype I served as the outgroup. All sequences were aligned by using ClustalX (www.ebi.ac.uk/clustalw), and phylogenetic analysis was performed by using MEGA 4.0 (www.megasoftware.net), according to the Tamura-Nei model. The reliability of the inferred phylogenetic tree was estimated by the bootstrap method, with 1,000 replications. Horizontal branch lengths are drawn to scale, and the tree was rooted by using the Asian genotype, which always appears as the most divergent. Scale bar represents percentage of genetic distance. Black circles represent sequences generated in this study and sequences from Rio de Janiero from 1998 and 2007–2008. The names of DENV-2 isolates include reference to year of isolation and country of origin: BR, Brazil; CO, Colombia; DO, Dominican Republic; EC, Ecuador; PR, Puerto Rico; PY, Paraguay; TH, Thailand; VE, Venezuela. A more detailed description of the methods used, as well as GenBank accession numbers for the isolates, can be found with the online version of this figure (www.cdc.gov/EID/content/16/3/576-F.htm)

2008 showed 6 aa substitutions in the envelope gene: V129I, L131Q, I170T, E203D, M340T, and I380V. Our results support the notion that aa positions at 129 and 131 in the envelope gene are critical genetic markers for phylogenetic classification of DENV-2 (*7*–*9*).

Notably, residue 131 in the envelope gene is located within a pH-dependent hinge region at the interface between domains I and II of the envelope protein. Mutations at this region may affect the pH threshold of fusion and the process of conformational changes (*10*).

Our results suggest the circulation of genetically different DENV-2 in Brazil and that these viruses may have a role in severity of dengue diseases. These findings can help to further understand the complex dynamic pathogenic profile of dengue viruses and their circulation in dengue-endemic regions.

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DOI: 10.3201/eid1603.090996

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Yersinia Species Isolated from Bats, Germany

To the Editor: Bats are distributed worldwide and are among the most diverse and species-rich mammals on earth. They exist in a large variety of distinct ecologic niches. Many bat species roost near humans, which is of particular interest for research on bat-to-human transmission of potential zoonotic pathogens. Moreover, migratory bats could act as long-distance vectors for several infectious agents. In recent decades, scientific interest in chiropteran species has markedly increased because bats are known hosts to zoonotic agents, such as henipaviruses, Ebola virus, and severe acute respiratory syndrome (SARS)–like corona viruses (*1*,*2*). However, investigations regarding bacterial pathogens with potential for mutual transmission between bats and humans are sparse. The effect of bacterial agents on individual bats is largely unknown and has been neglected in most studies published to date (*3*).

We conducted a broad study during 2006–2008 on diseases and causes of death in bats among 16 species found in Germany. Two hundred deceased bats, collected in different geographic regions in Germany (southern Bavaria, eastern Lower Saxony, eastern Brandenburg, and Berlin greater metropolitan area), were subjected to necropsy and investigated by using routine histopathologic and bacteriologic methods. During necropsy, instruments were dipped in 70% ethanol and moved into a Bunsen burner flame after every incision to prevent any cross-contamination. For bacteriologic examination, tissue samples were treated accordingly to prevent environmental contamination. A freshly cut tissue surface was plated onto Columbia agar (5% sheep blood; Oxoid, Wesel, Germany), Gassner agar (Oxoid), and MacCo-

nkey agar (Oxoid) and incubated at 37°C for 24–48 hours.

Twenty-five bacterial genera were cultured from bats, including 2 known human-pathogenic *Yersinia* spp., i.e., *Y. pseudotuberculosis* and *Y. enterocolitica*. The first *Yersinia* strain (Y938) was cultured from lung, heart, kidney (pure cultures), liver, spleen, and intestine (mixed cultures) of a greater mouse-eared bat (*Myotis myotis)*. This isolate was identified as *Y. pseudotuberculosis* by Api 20E (bioMérieux, Nürtingen, Germany), Micronaut-E (Merlin Diagnostik GmbH, Bornheim-Hersel, Germany), and 16S rRNA gene analysis (Table). The sequence was deposited into GenBank under accession no. FN561631. Further serologic characterization by agglutination test (Denka Seiken, Tokyo, Japan) and multilocus sequence typing (*4*) identified *Y. pseudotuberculosis* serogroup 1, biovar 5, sequence type (ST) 43 in all tissue samples investigated. During necropsy, severe enlargement of the liver and a marked hemoperitoneum were seen. Microscopic examination showed multifocal severe necrotizing hepatitis and splenitis associated with numerous intralesional gram-negative coccobacilli and a moderate to marked interstitial pneumonia. The remaining organs, including heart, kidney, and intestine, had no pathologic changes.

The second *Yersinia* strain (Y935), *Y. enterocolitica*, was isolated in pure culture from spleen and intestine of a common pipistrelle (*Pipistrellus pipistrellus*) and identified by the methods described above (Table). The 16S rRNA sequence was deposited into GenBank under accession no. FN561632. No bacteria were cultured from any other organ. Based on results of an agglutination test (Denka Seiken), the isolate was characterized as *Y. enterocolitica* serotype O:6, biovar 1A. Necropsy and histopathologic examination showed no inflammatory changes, suggesting a subclinical state of infection.

Yersiniosis is a bacterial disease with a wide distribution and host range. *Y. pseudotuberculosis* and *Y. enterocolitica* are frequently isolated from a variety of wild and domestic animals (*5*), but little is known about the occurrence of yersiniosis in free-ranging chiropteran species. Only few reports of fatal *Y. pseudotuberculosis* infection in captive flying foxes have been published (*6*,*7*). In Europe, *Y. pseudotuberculosis* strains belonging to serogroup 1 are most common and cause most *Y. pseudotuberculosis* infections in humans and animals (*5*). Isolates of ST43 in the multilocus sequence typing database (*4*) came from humans, birds, hares, hedgehog, cat, dog, and pig in Europe; humans in Asia; marsupial in Australia; and deer in Australia and New Zealand. We report an isolate from a free-ranging bat in Germany. *Y. enterocolitica* biovar 1A has been found in a wide range of human, animal, and environmental sources. Although often considered nonpathogenic, this biovar is described as an opportunistic pathogen (*8*), and serovar O:6 has been detected as the causative agent of ovine placentitis and abortion (*9*).

Transmission of both *Yersinia* species generally occurs after ingestion of contaminated food or water. All bat species in Germany are insectivorous, and insects can be infected with various microbial agents. Investigations concerning bacteria–insect interactions showed that insects may carry pathogenic bacteria, including *Yersinia* (*10*); thus, insects or contaminated water are possible sources of both species described.

In conclusion, *Y. pseudotuberculosis* and *Y. enterocolitica* were isolated from 2 bat species in Germany, representing evidence of *Yersinia* spp. in free-ranging vespertilionids. Histopathologic findings of the greater mouse-eared bat were consistent with those of systemic *Y. pseudotuberculosis* infection, rendering this species pathogenic for bats. The common pipistrelle was subclinically infected with *Y. enterocolitica*. The role of wild animals as reservoir hosts for bacterial pathogens such as *Yersinia* spp. is well known, underlining the need for biologists and persons handling wildlife to be aware of these zoonotic infectious agents.

Table. Strain typing results of *Yersinia* spp. isolates from free-ranging bats, Germany*

Method	*Y. pseudotuberculosis*†	*Y. enterocolitica*‡
Api 20E profile§	1 0 1 4 1 1 2 1 7	1 1 5 4 7 2 3 1 7
Micronaut E,¶ % probability	100% *Y. pseudotuberculosis*	99.77% *Y. enterocolitica*
16S rRNA gene analysis, % similarity to sequences in GenBank	100% (1,058 bp) to *Y. pseudotuberculosis* YPIII#	100% (985 bp) to *Y. enterocolitica* strain 280**
Serologic characterization by agglutination test	Serogroup 1, biovar 5	Serotype O:6, biovar 1A
Multilocus sequence typing††	1 2 1 2 5 2 1	Not determined

**Y. pseudotuberculosis* obtained from lung, heart, kidney, spleen, liver, and intestine; *Y. enterocolitica* obtained from spleen and intestine. For *Y. pseudotuberculosis* spleen, liver, and intestine samples, isolates were obtained in mixed culture accompanied by colonies of 1–2 nonspecific bacteria. For all remaining tissues, *Y. pseudotuberculosis* and *Y. enterocolitica* were isolated in pure culture.
†GenBank accession no. FN561631.
‡GenBank accession no. FN561632.
§bioMérieux, Nürtingen, Germany.
¶Merlin Diagnostik GmbH, Bornheim-Hersel, Germany.
#GenBank accession no. CP000950.
**GenBank accession no. FJ641888.
††Of gene loci *adk*, *argA*, *aroA*, *glnA*, *thrA*, *tmk*, *trpE.*

Acknowledgments

We thank Nadine Jahn, Doris Krumnow, Stephan Schatz, and Robert Schneider for excellent technical assistance and Martin Pfeffer and Holger Scholz for editorial help.

This study was supported by the Adolf and Hildegard Isler Stiftung and the Klara Samariter Stiftung. The multilocus sequence typing database is publicly available at http://mlst.ucc.ie, which is currently supported by a grant from the Science Foundation of Ireland (05/FE1/B882).

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DOI: 10.3201/eid1603.091035

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Human Herpesvirus 8, Southern Siberia

To the Editor: Human herpesvirus 8 (HHV-8) is the etiologic agent of Kaposi sarcoma. Sequence analysis of the highly variable open reading frame (ORF)–K1 of HHV-8 has enabled the identification of 5 main molecular subtypes, A–E (*1*). A and C subtypes are prevalent in persons in Europe, Mediterranean countries, northwestern China, and the United States; subtype B, in persons in sub-Saharan Africa; subtype D, in persons in the Pacific Islands and Japan (*2*–*6*); and subtype E, in Native Americans in the United States.

Considering that K1 gene polymorphisms of HHV-8–infected persons reflect the divergence accumulated during the early migrations of modern humans out of Africa (*1*), it is tempting to put the polymorphisms observed in the different subtypes into an evolutionary perspective with their geographic distribution. It is thought that Native Americans infected by subtype E and Pacific Islanders, including those infected by subtype D in the Japanese archipelago, originated from a common ancestral genetic stock in continental Asia. Because Siberia constitutes the geographic link between mainland Asia, North America, and the Pacific (online Technical Appendix, www.cdc.gov/eid/content/16/3/585-Techapp.pdf), it is likely that the Siberian region has served as a source or a corridor of human dispersals to these regions. Thus, we conducted a molecular epidemiology HHV-8 survey of the Buryat population, a major indigenous group in southern Siberia, to gain new insights into the origins, possibly common, of HHV-8 subtypes D and E.

After consent of local authorities and participants, we collected 745 human blood samples in 1995 in 17 medicosocial structures (homes for elderly persons, veterans of the Russian army,

hospitalized persons, blood donors) located near Lake Baïkal and originating from Ulan Ude (344), Ust Orda (216), and Chita (185), Siberia, Russia (additional data can be obtained directly from the authors). The median age of those included was 52 years (range 25–98 years); 489 (66%) were women. Antibodies against HHV-8 latency–associated nuclear antigen were identified by immunofluorescent antibody assay by using the BC3 cell line (*3*). Punctuate nuclear staining of BC3 cells at a 1:160 dilution was observed for 187 (25.1%) patients with no difference according to investigated regions ($p = 0.32$ by χ^2 test) or between men (25.8%) and women (24.7%) ($p = 0.76$ by χ^2 test; online Technical Appendix). However, HHV-8 seroprevalence increased with patient age, rising from 12.9% (25–43 years) to 46.4% (≥61 years) ($p = 1.8 \times 10^{-13}$ by χ^2 test for trend) (Figure; online Technical Appendix). No significant difference was observed in antibody titers according to age ($p = 0.45$ by Fisher exact test). These results demonstrate that HHV-8 infection is highly prevalent in the Siberian adult population tested.

HHV-8 infection was determined by nested PCR that amplified a 737-bp fragment of the ORFK1 in peripheral blood buffy coats of 85 HHV-8–seropositive and 10 HHV-8–seronegative persons (*3*). Amplification was positive in 19/85 (22.4%) samples; sequences were obtained for 18 of these samples (online Technical Appendix). These sequences showed 0%–7.31% nucleotide divergence and 0%–3.55% amino acid divergence. Nevertheless, 17 strains were found to be closely related with <1.75% nucleotide differences for 684 nt, and only 1 sequence (1445 strain) displayed higher nucleotide divergence.

A comparative sequence analysis, including 66 representatives of K1 gene sequences of the HHV-8 A/C subtypes/subgroups, and sequences obtained from persons originating from Russia, was performed (*7*–*9*). Seventeen of the 18 HHV-8 strains from Siberia belonged to the A subtype; 15 clustered in a newly identified specific subclade (online Technical Appendix). Notably, the 1445-Siberian strain, which exhibits the typical 5 aa deletion at positions 201–205, belongs to subtype C and clustered with the 7848 strain previously described by Lacoste et al. (*9*). Furthermore, both strains originate from Chita.

Our results indicate that HHV-8 infection is highly prevalent in the population tested in southern Siberia and extend current knowledge on the worldwide distribution of HHV-8 genotypes. The presence of a Siberian strain monophyletic subclade suggests the existence of HHV-8 strains preferentially spreading among this population in southern Siberia.

To ascertain the maternal ancestry of these persons, we sequenced the hypervariable region I (HVS-I) of the maternally-inherited mitochondrial DNA (mtDNA) and assigned haplogroups on the basis of the HVS-I motifs. Our analyses showed that 17/18 persons analyzed showed a mtDNA motif of clear continental east Asian origin (e.g., A, D correspond to different mtDNA haplogroups). One person (1474-strain) had a lineage (i.e., HV1) that is thought to have a western Eurasian origin. Overall, these mtDNA analyses indicate that the maternal ancestry of the persons examined here can be unambiguously attributed to East Asia, and not to Western Eurasia. K1 subtype A sequences recently found in the Xinjiang Uygur region in China (*10*) do not correspond to the specific Siberian clade described in our study. Thus, we must now consider that the widely distributed HHV-8 A/C subtype, so far mainly observed in Europe and Mediterranean countries, is also largely predominant in continental Asia.

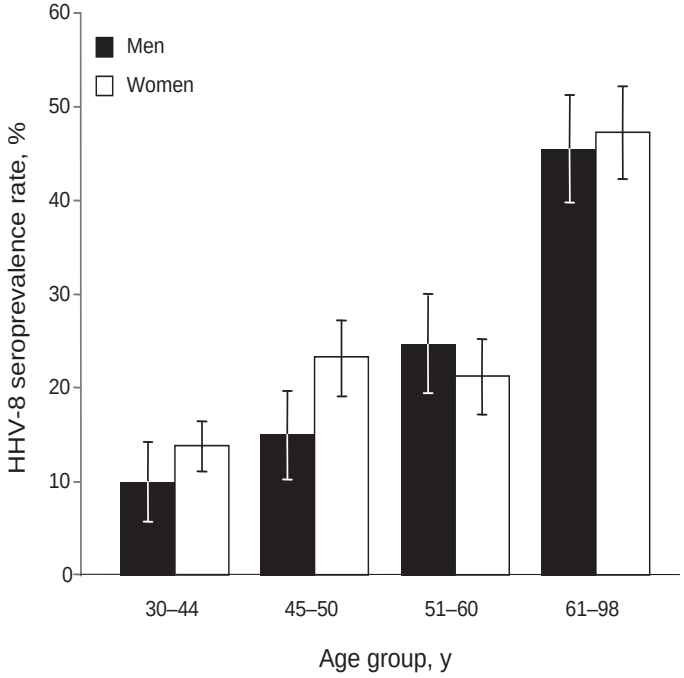

Figure. Age-dependent human herpesvirus 8 (HHV-8) seroprevalence rates for 745 persons in southern Siberia 25–98 years of age who lived in the Ust Orda, Ulan Ude, or Chita districts during 1995. Seropositivity was based on strict criteria; only samples showing punctuate nuclear staining clearly reactive at a dilution ≥1:160 were considered HHV-8 positive. All 187 HHV-8–seropositive samples were tested for antibodies directed against HIV-1/2 by using Genscreen HIV-1/2 Antibody Assay (Bio-Rad Laboratories, Marnes-la-Coquette, France); only 2 were seropositive. Error bars indicate 95% confidence intervals.

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DOI: 10.3201/eid1603.091390

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Sus-Pense

R.L. Bernstein

Don't call it swine flu:
if you dine on pork, you
can't catch the flu, as we know.
But the pork people say
some customers may
be confused by the name and just go.
Now look at the news:
around the world views
show people with a mask on their face.
Despite the name change,
folks who find H1N1 strange
still want to keep swine in their place.
For me it is clear:
I don't have the fear
of swallowing bacon-wrapped figs.
While others may hurry
to wear masks, I don't worry.
I really don't plan to kiss pigs.

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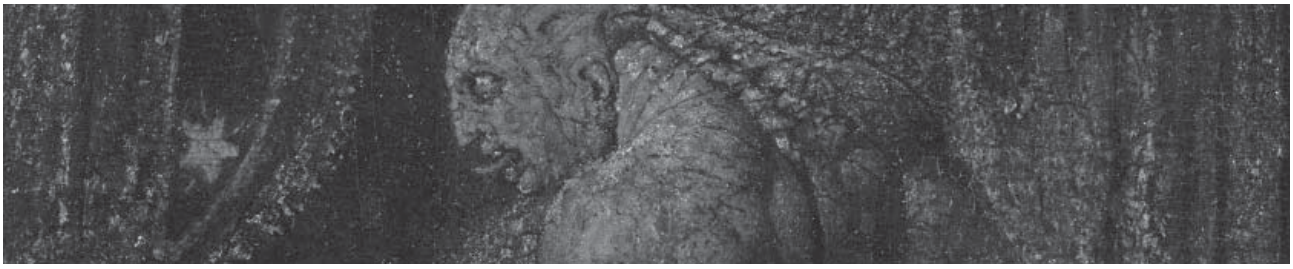

William Blake (1757–1827) The Ghost of a Flea (1819–20) Tempera mixture panel with gold on mahogany (21.4 cm × 16.2 cm) Tate, London, England

...a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em,
And so proceed *ad infinitum*

—Jonathan Swift, On Poetry: a Rhapsody (1733)

Polyxeni Potter

"There was no doubt that this poor man was mad, but there is something in the madness of this man which interests me more than the sanity of Lord Byron and Walter Scott," remarked William Wordsworth about his fellow poet William Blake. Blake's own claims to outlandish visions added fuel to rumors of his insanity. As a mere child, he saw God "put his head through the window" and on another occasion, "a tree filled with angels, bright angelic wings bespangling every bough like stars." Later in life, when faced with the death of a younger sibling, he saw this brother's spirit "clapping its hands for joy."

Blake was a Londoner, born in a spacious old home at 28 Broad Street, Golden Square, the son of a hosier. He never went to school and throughout his life was glad to have escaped formal education, "Improvement makes strait roads; but the crooked roads without Improvement are roads of genius." His family indulged his talents. "As soon as the child's hand could hold a pencil it began to scrawl rough likenesses of man or beast and make timid copies of all the prints he came near." At age 10 he was sent to a fashionable preparatory school for young artists, and at 14, he was apprenticed to Basire, engraver to the Society of Antiquaries, who sent him to draw old monuments, especially at Westminster Abbey. His love of Gothic art dates from this time.

He delighted in the linear nature of monuments for he believed "firm and determinate lineaments unbroken by shadows" to be the essence of art. He abhorred chiaroscuro, the art of Venice and Flanders, because its interplay of light and shadow blurred outlines. Linear style was also characteristic of religious art. The spirits he drew, Blake insisted, should be "organized" within determinate and bounding form. Admiration of Greek antiquities and mythology also nurtured his style and subject matter.

For a man who never strayed far from his home town, he became very cultured in the visual arts. In his prime a distinguished printer, painter, poet, and musician, as well as prophet and iconoclast, Blake took education in his own hands. He learned Greek, Latin, and Hebrew to appreciate the classics in the original and invented his own engraving and color preservation techniques. He combined his facility with the word and brush in "illuminated printing," a technique rooted in the Middle Ages, to bring poetry to the reader through the eye of the poet's own imagination. Eccentric and nonconformist, he associated with radical thinkers, among them Thomas Paine and Mary Wollstonecraft.

Blake's work was on a small scale and often contained in the pages of books. But his imaginings were boundless. "He who does not imagine in stronger and better lineaments and in stronger and better light than his perishing and mortal eye can see does not imagine at all." To him the great art of the world depicted not that seen by the "mortal eye" but a more perfect imagined form. His idiosyncratic approach to life and the individuality of his craft defy labels. While his work places him solidly among the Romantics, some have labeled him a forerunner of modern anarchism.

Many spirits or ghosts Blake drew seemed to derive from his Gothic studies; others were of kings or queens.

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DOI: 10.3201/eid1603.000000

The ghost of a flea, on this month's cover, seems that of a demon. This miniature was part of a series of "visionary heads," commissioned by his friend John Varley, landscape painter and astrologer, who believed in spirits but was unable to see them. He was drawn to Blake, who professed to live with them. The two would meet and try to summon spirits of historical or mythologic figures and if they appeared, Blake would draw them. They were angels, Voltaire, Moses, and the flea, which told them that "Fleas were inhabited by the souls of such men as were by nature blood thirsty to excess."

"I called on him one evening and found Blake more than usually excited," Varley reported, "He had seen a wonderful thing—the ghost of a flea!" "And did you make a drawing of him?" Varley asked. "I wish I had," Blake responded, "but I shall, if he appears again! ...There he comes! His eager tongue whisking out of this mouth…." Varley gave him paper and a pencil to draw the portrait. "I felt convinced by his mode of proceedings that he had a real image before him, for he left off, and began on another part of the paper, to make a separate drawing of the mouth of the flea, which the spirit having opened, he was prevented from proceeding with the first sketch, till he had closed it."

Varley described the conception as a "naked figure with a strong body and a short neck—with burning eyes which long for moisture, and a face worthy of a murderer holding a bloody cup in its clawed hands, out of which it seems eager to drink…. I never saw any shape so strange, nor did I ever see any colouring so curiously splendid—a kind of glistening green and dusky gold, beautifully varnished."

Both in his poetry and his art, Blake often personified death, war, famine, and other abstractions, ascribing them faces and human characteristics. The ghost of his flea is muscular, part human part reptile, loaded with symbolic clues of its nature and character. The creature strides theatrically across a stage framed by opulent drapes and sprinkled with stars—Blake's friend and supporter John Linnell made a copy of this drawing for Zodiacal Physiognomy, as a sign of Gemini.

The left hand holds an acorn, the right a thorn. The massive frame scarred by a protruding spine supports a small head, vaguely alluding to the shape of a flea. On the floor near the feet, an insect, the physical embodiment, completes the portrait. Despite claims to a visionary source, this flea recalls the imps of Henry Fuseli (1741–1825), another painter of monsters, and some of Blake's previous work. It could also have been informed by the drawings of early microscopist Robert Hooke (1635–1703), whose illustration of a flea in his book Micrographia described it as "adorn'd with a curiously polish'd suite of sable Armour, neatly jointed …."

"It's God. / I'd know him from Blake's picture anywhere," Robert Frost's Eve said in "Masque of Reason." Whether he was drawing the Almighty or a tiny insect, Blake captured and uncloaked the unadulterated character of the subject. And whatever the source of his inspiration, it lit and portrayed this character in all its purity.

Not fooled by the tiny creature he tossed on the scene as a reference, Blake knew and spelled out its horrific nature. And he was not alone. The flea was notorious for its pestiferous qualities. They did not escape the attention of Jonathan Swift: "The vermin only tease and pinch / Their foes superior by an inch." John Donne (1572–1631) had exploited the flea's blood-drinking habits in his immortal plea to a mistress, acknowledging the importance of fluid exchange, before the possibility of contagion even entered the equation, "Me it suck'd first, and now sucks thee, / And in this flea our two bloods mingled bee."

The perpetual struggle against these pests inspires poetry to this day: "Fleas / Adam / had'em." The incongruous imbalance between their size and their impact on humanity, shown in no less than their deadly connection with the history of Black Death, is now fully understood. Fleas are known for the vectors of disease they are, spreading in addition to bubonic plague, murine or fleaborne typhus and other rickettsioses in new areas, and still tormenting and killing humans, despite improved diagnostic techniques and treatments. Swift would see no humor in the *ad infinitum* emergence of novel spotted fever strains causing disease in yet more areas. He could not have known that microorganisms, such as rickettsia, infect fleas and through them spread to other animals, in fact becoming what he had lightly referred to as the fleas of fleas.

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EMERGING INFECTIOUS DISEASES

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Community-associated Methicillin-Resistant *Staphylococcus aureus* Strains in Pediatric Intensive Care Unit

Household Transmission of Pandemic (H1N1) 2009, San Antonio, Texas, USA

Influenza-associated Acute Respiratory Distress Syndrome

Contribution of *Streptococcus anginosus* to Group C and G Streptococcal Infections, South India

Escherichia albertii in Wild and Domestic Birds

Clostridium difficile Infection among Hospitalized Children, United States, 1997–2006

Enterohemorrhagic *Escherichia coli* O157, Germany, 1987–2008

Genotype Profiles to Differentiate Sources of Foodborne Outbreaks

Alfred Russel Wallace and the Anti-Vaccination Movement in Victorian England

Pigs as Reservoir for Gentamicin-Resistant *Enterococcus faecalis*

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Pandemic (H1N1) 2009 in Breeding Turkeys, Valparaiso, Chile

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Complete list of articles in the April issue at http://www.cdc.gov/eid/upcoming.htm

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Fifth Decennial: International Conference on Healthcare-Associated Infections 2010
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International Conference on Emerging Infectious Diseases 2010
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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscapecme.com/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Extensively Drug-Resistant *Mycobacterium tuberculosis* from Aspirates, Rural South Africa

CME Questions

1. Which of the following statements about tuberculosis (TB) infection among patients with concomitant HIV infection is most accurate?

A. Smear-positive pulmonary disease and extrapulmonary TB are more common
B. Smear-positive pulmonary disease is more common, but extrapulmonary TB is less common
C. Smear-negative pulmonary disease and extrapulmonary TB are more common
D. Smear-negative pulmonary disease is more common, but extrapulmonary TB is less common

2. Which of the following statements about patients participating in the current study is most accurate?

A. Less than 20% were HIV positive
B. Pleural fluid cultures were much more common than lymph node cultures
C. Most patients had a history of TB
D. Approximately one third of lymph node and pleural fluid cultures grew TB

3. In what percentage of patients was the positive lymph node or pleural fluid aspirate culture the only source of a culture diagnosis of TB?

A. 5%
B. 20%
C. 45%
D. 75%

4. Which of the following statements about the diagnosis of extensively drug-resistant (XDR) TB is most accurate?

A. More than half of cases of XDR TB were diagnosed by fluid aspirate alone
B. XDR TB was more common in lymph node vs pleural fluid aspirate cultures
C. XDR TB was not discovered in any lymph node aspirate culture
D. There was 100% concordance between the growth of XDR TB in sputum and pleural fluid cultures

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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The journal 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, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, 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 email 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. 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.

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Types of Articles

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), a one-sentence summary of the conclusions, and a brief biographical sketch. 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), a one-sentence summary of the conclusions, and a brief biographical sketch. 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), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. 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), a one-sentence summary of the conclusions, 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 no more than 1,200 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 2); tables (not to exceed 2); and a brief biographical sketch. 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 no 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. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.