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Tuberculosis

March 2014

David Flores (1973– ) Nelson Mandela Mural (2013) Spray paint on building (14 × 50 ft) Venice, California

# EMERGING INFECTIOUS DISEASES®





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March 2014


## On the Cover

**David Flores (1972– )**

***Nelson Mandela Mural* (2013)**

**Spray paint on building (14′ × 50′)**
**Venice, California**

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## Synopsis

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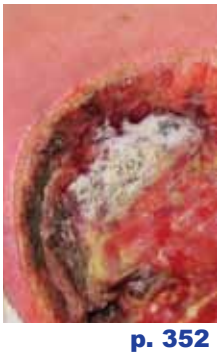

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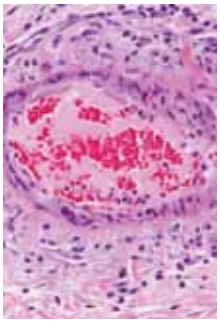



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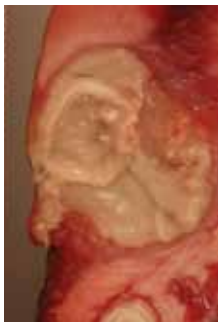



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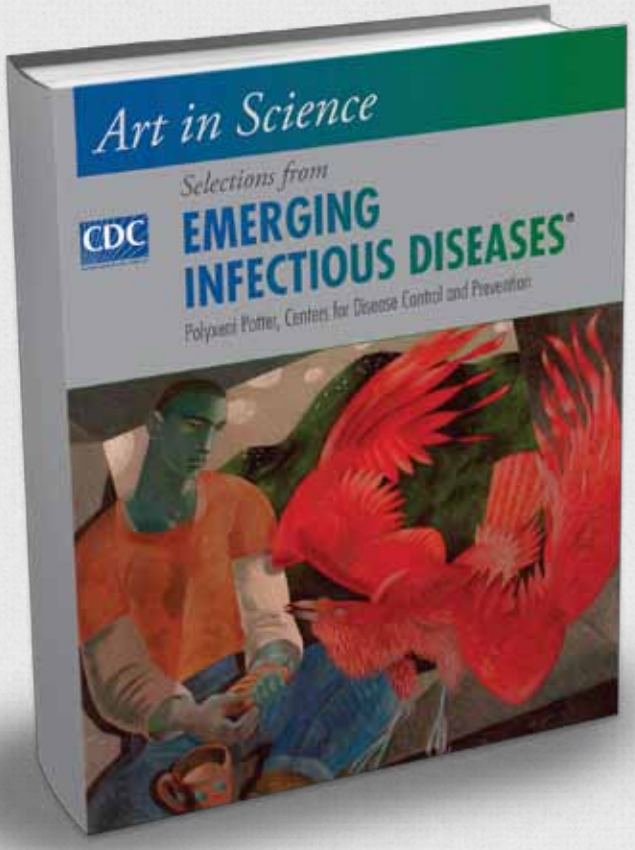


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# Invasive Fungal Infections after Natural Disasters

**Kaitlin Benedict and Benjamin J. Park**

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**Release date: February 13, 2014; Expiration date: February 13, 2015**

**Learning Objectives**

Upon completion of this activity, participants will be able to:

- Analyze the risk for fungal infections after natural disasters, including coccidioidomycosis
- Distinguish features of water-related fungal infections after natural disasters
- Assess the prevalence and microbiological characteristics of soft tissue fungal infections after natural disasters
- Evaluate the health risks associated with exposure to mold after natural disasters.

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**Authors**

*Disclosures:* ***Kaitlin Benedict, MPH,*** *and* ***Benjamin J. Park, MD,*** *have disclosed no relevant financial relationships.*

The link between natural disasters and subsequent fungal infections in disaster-affected persons has been increasingly recognized. Fungal respiratory conditions associated with disasters include coccidioidomycosis, and fungi are among several organisms that can cause near-drowning pneumonia. Wound contamination with organic matter can lead to post-disaster skin and soft tissue fungal infections, notably mucormycosis. The role of climate change in the environmental growth, distribution, and dispersal mechanisms of pathogenic fungi is not fully understood; however, ongoing climate change could lead to increased disaster-associated fungal infections. Fungal infections are an often-overlooked clinical and public health issue, and increased awareness by health care providers, public health professionals, and community members regarding disaster-associated fungal infections is needed.

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The potential for adverse health events after natural disasters is well recognized and comprises various challenges for public health (*1*). The World Health Organization defines a disaster as a disruption of society resulting in widespread human, material, or environmental loss that exceeds the affected society's ability to cope by using local resources (*2*). Natural disasters can be broadly classified into 3 groups: geophysical (e.g., earthquakes, volcanic eruptions, and tsunamis), hydrometeorological (e.g., floods, hurricanes, and tornadoes), and geomorphological (e.g., landslides and avalanches). Specific social, economic, and cultural settings create a unique set of circumstances for every disaster, and the immediate causes of illness and death (such as blunt trauma, lacerations, crush injuries, suffocation, and drowning) vary according to the type of event (*1*).

Infectious disease outbreaks after natural disasters are uncommon. However, features of the post-impact and recovery phases of disasters, such as population displacement, low vaccine coverage for vaccine-preventable diseases, inadequate sanitation and hygiene infrastructure, and limited access to health care services, can interact to increase the risk for transmission of infectious diseases that were previously established in the affected area (*3*). Disaster-associated fungal infections are similarly uncommon, but they are becoming increasingly recognized and are typically attributable to the impact phase of a disaster because such infections primarily result from inhalation or cutaneous inoculation of fungal spores directly from the environment (*3*,*4*). During a disaster, pathogenic fungi can be displaced from their natural habitats, which could increase their environmental concentration or introduce them to areas where they would not normally be found, resulting in contact with injured persons and potentially causing fungal infections. To increase awareness of these events among health care providers and public health officials, we summarize the known occurrences of fungal infections associated with natural disasters (Table).

### Search Strategy and Selection Criteria

The online literature databases PubMed and Google Scholar were searched for English-language articles about fungal infections related to natural disasters that were published as of April 2013. Search terms included combinations of "disaster," "natural disaster," "tornado," "hurricane," "earthquake," "tsunami," and "flood" with "fungal infection,"

Table: Disaster-associated fungal infections*

| Disaster | Reference | Location | No. cases | Fungal organism | Type of infection | Outcome |
|---|---|---|---|---|---|---|
| Tornado, 2011 | Neblett Fanfair et al. (*4*) | USA | 13 | *Apophysomyces trapeziformis* | Soft tissue | 38% all-cause mortality |
| Great East Japan Earthquake and Tsunami, 2011 | Kawakami et al. (*5*) | Japan | 1 | *Aspergillus fumigatus* | Pulmonary, multi-organ dissemination | Death |
| | Nakamura et al. (*6*) | Japan | 1 | *Scedosporium apiospermum* | Lung and brain abscesses | Death |
| | Igusa et al. (*7*) | Japan | 1 | Pathogen not identified† | Sinusitis and meningitis | Death |
| Hurricane Ike, 2008 | Riddel et al. (*8*) | USA | 3 | Unspecified agent of chromoblastomycosis | Soft tissue | Recovery |
| Hurricane Katrina, 2005 | Rao et al. (*9*) | USA | 1 | *Cladosporium* sp. | Pulmonary | Resolved without treatment |
| Indian Ocean Tsunami, 2004 | Petrini et al. (*10*) | Thailand | 2 | *Cladophialophora bantiana* | Soft tissue | Recovery |
| | Garzoni et al. (*11*) | Thailand | 2 | *Scedosporium apiospermum* | Spondylodiscitis, 1; brain abscess,1 | Recovery |
| | Gunaratne et al. (*12*) | Colombo, Sri Lanka | 6 | *A. fumigatus* | Meningitis | 50% all-cause mortality |
| | Andresen et al. (*13*) | Sri Lanka | 1 | *Apophysomyces elegans* | Soft tissue | Not specified |
| | Snell and Tavakoli (*14*) | Thailand | 1 | *A. elegans* | Soft tissue | Recovery |
| | Maegele et al. (*15*) | Southeast Asia | 1<br>1 | *Fusarium* sp.<br>*Mucor* sp. | Soft tissue, sepsis<br>Soft tissue | Death<br>Not specified |
| Earthquake, 1994 | Schneider et al. (*16*) | USA | 203 | *Coccidioides immitis* | Pulmonary; 6 (3.7%) disseminated | 1.5% all-cause mortality |
| Volcano, 1985 | Patiño et al. (*17*) | Colombia | 8 | *Rhizopus arrhizus* | Soft tissue | 80% all-cause mortality |
| Dust storm originating near Bakersfield, California, 1977 | Flynn et al. (*18*) | USA | 115 | *C. immitis* | Pulmonary; 16 (14%) disseminated | 7% all-cause mortality |
| | Williams et al. (*19*) | USA | 18 | *C. immitis* | Pulmonary; 4 (22%) disseminated | 5.5% all-cause mortality |

*As documented in reports with sufficient detail about the number of persons affected, pathogen, and body site. Reports describing cases of post-disaster fungal colonization without infection are not included in the table. Number of cases and percentages are provided when data were available.
†Fungal infection diagnosed on the basis of cerebrospinal fluid profile (decreased glucose, high mononuclear cell count, + β-D glucan test result).

"fungal disease," "fungus," or "mold." References cited in relevant articles were also reviewed, and the authors' personal records were searched for conference abstracts.

### Pathogenic Fungi and the Environment

Of the 1.5 million species of fungi on Earth, ≈300 are known human pathogens (*20*). Pathogenic fungi exist in a broad range of natural habitats but are believed to be more common in subtropical and tropical areas of the world, probably because of environmental restrictions on their growth or propagation (*20*). Known geographic habitats of some pathogenic fungi (for example, *Blastomyces*, *Coccidioides*, and *Histoplasma*) are well defined, but others (such as *Aspergillus* and other molds) are thought to be ubiquitous. The abundance and distribution (and therefore, potential to cause disease) of environmental fungi probably depend on climatic or environmental factors such as ambient temperature and moisture (*20*). Examples include coccidioidomycosis incidence in Arizona, which has been shown to correlate with hot, dry conditions; blastomycosis clusters observed in association with rainfall after periods of decreased precipitation; and incidence of *Penicillum marneffei* penicilliosis, an opportunistic infection which is endemic to Southeast Asia and increases in incidence during rainy months (*21*,*22*). Aspergillosis and other invasive mold infections have also been postulated to correlate with seasons or weather patterns in some geographic areas (*23*).

Although seasonal changes and weather effects probably play a role in the growth and distribution of many pathogenic fungi, environmental disruption is a key factor in the dispersal of these organisms and their resulting potential for causing infection. Both small-scale earth-disrupting activities, such as excavation or construction, and events affecting larger areas, such as earthquakes (*16*), tsunamis (*5–7*,*10–15*,*24*,*25*), and tornadoes (*4*), have been linked to the occurrence of fungal infections. Natural disasters can cause large-scale disruption of fungal habitats, which can lead to clusters of respiratory, cutaneous, or other forms of fungal disease.

Studies of clinical specimens collected from persons injured during disasters highlight the diversity of potential fungal pathogens in the natural environment. For example, after an 8.0-magnitude earthquake in Wenchuan, China, during 2008, 19 strains of fungi were identified in wound, sputum, and blood cultures from 123 injured persons (*26*), and fungi accounted for 7.6% of clinical isolates obtained from 42 patients with crush syndrome (*27*). Similarly, after a 1970 tornado in Texas, United States, fungi were identified in 8 (6.5%) of 124 wound isolates from 24 hospitalized patients (*Fusarium*, unspecified yeast, *Rhodotorula*, *Aspergillus*, *Hormodendrum* [now *Cladosporium*], and *Cephalosporium*), and in 4 (10.5%) of 38 wound isolates from 23 ambulatory patients (*28*). In each of these reports, multiple organisms were isolated from many of the patients, and the fungi recovered may not necessarily have been agents of infection.

### Respiratory Fungal Infections after Disasters

#### Airborne

Inhalation is a common route for fungal infections. Fungi are known to cause respiratory infections ranging from asymptomatic to life-threatening, depending on the pathogen and host characteristics. *Coccidioides* spp. are dimorphic fungi that grow in semiarid soil and are endemic to the southwestern United States, northern Mexico, and parts of South America. Two instances of disaster-associated coccidioidomycosis have been described.

An outbreak of coccidioidomycosis after the January 1994 earthquake in Northridge, California, United States, was 1 of few known examples of any infectious disease outbreak directly related to a geophysical disaster (*3*). *Coccidioides* spores were presumably aerosolized as a consequence of the earthquake, its aftershocks, and associated landslides and were dispersed by the resulting widespread dust clouds (*16*). In Ventura County, California, 203 outbreak-associated coccidioidomycosis cases were identified, and investigators found that dust exposure was substantially associated with acute illness (*16*). Fungal infection may not have been considered in the initial diagnoses in this outbreak; 93% of case-patients received ≥1 antibacterial drug before coccidioidomycosis was diagnosed (*16*).

Another coccidioidomycosis outbreak occurred after a severe dust storm in the southern San Joaquin Valley of California in December 1977 (*18*). The storm originated near Bakersfield, an area to which coccidioidomycosis is highly endemic, and covered nearly 90,000 $km^2$, an area larger than the state of Maine (*18*). In Sacramento County, an area to which the disease was not previously considered to be endemic, 115 cases of coccidioidomycosis were attributed to the dust storm, including 16 cases of disseminated disease (*18*). Eighteen additional cases were identified at a US Navy air station in Kings County (*19*), and other California counties affected by the storm saw more coccidioidomycosis cases than usual; for example, Kern County recorded 134 cases during January and February 1978, compared with 17 cases during those months in the previous year (*29*).

#### Near-drowning

Drowning and near-drowning are common during and after disaster-related flooding (*1*). Aspiration of contaminated or debris-laden water can lead to sinus and pulmonary infections; aspiration pneumonia is often referred to as "tsunami lung" in post-tsunami settings (*16*,*24*). Tsunami lung can be caused by bacteria, fungi, or both.

*Pseudallescheria boydii* (asexual form, *Scedosporium apiospermum*) is hypothesized to be the most common fungal pathogen associated with near-drowning, although this finding has not been studied specifically in the context of disasters (*30*). Information about post-disaster *Scedosporium* lung infection is limited to a small number of case reports; these reports also document the organism's propensity to progress to central nervous system infection, even in immunocompetent hosts (*6*,*11*). Other fungal pathogens, such as *Aspergillus*, have also been implicated as agents of tsunami lung; after the 2011 Great East Japan Earthquake and subsequent tsunami, a previously healthy near-drowning victim who later died was found to have pneumonia caused by *Aspergillus fumigatus* and evidence of multiorgan disseminated aspergillosis upon autopsy (*5*). The report describes delays in specimen transportation and receipt of culture results caused by the aftermath of the earthquake, which led to a delay in diagnosis and treatment (*5*).

Evidence of tsunami lung also was also apparent after the December 2004 earthquake and tsunami in the Indian Ocean, which killed >200,000 persons (*3*). In Sri Lanka, acute respiratory issues attributed to post-aspiration pneumonitis and polymicrobial pneumonia that were not related to communicable illnesses were the most frequent medical problems after this disaster (*31*). In Banda Aceh, Indonesia, several patients with necrotizing pneumonia, who did not respond to broad-spectrum antibacterial drugs, probably had polymicrobial infections that may have included fungal organisms (*24*). However, limited diagnostic capacity for fungi may have affected the ability to identify the potential role of fungi in these infections. A report from Germany demonstrated that among a cohort of 17 tourists injured during the tsunami, all had clinical and radiologic evidence of aspiration pneumonitis and pneumonia; *Candida albicans* and *A. fumigatus* were isolated from the respiratory tract of several patients, although it is unclear whether the isolation of these organisms represented true infection or colonization (*15*).

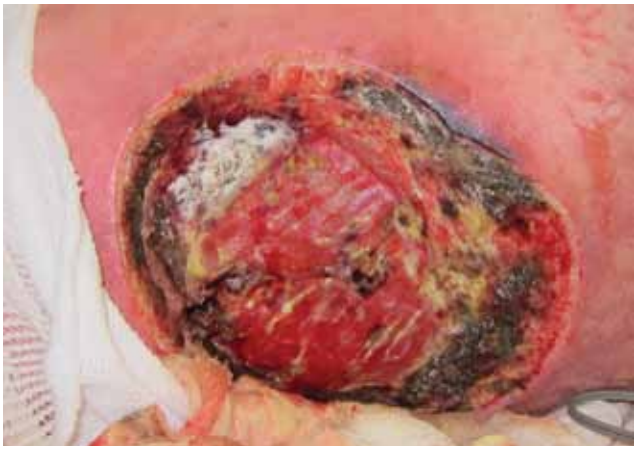

Figure. Necrotizing cutaneous mucormycosis, Joplin, Missouri, USA, 2011 (*4*). A left flank wound in a mucormycosis case-patient, with macroscopical fungal growth (tissue with white, fluffy appearance) and necrotic borders before repeated surgical debridement. Copyright 2012 Massachusetts Medical Society. Reprinted with permission.

### Soft Tissue Fungal Infections after Disasters

The risk for wound infections after a natural disaster is high when wounds are contaminated with water, soil, or debris (*32*). In addition, damage to the local health care infrastructure can compromise the ability to properly irrigate contaminated wounds with sterile solution or promptly treat injured persons with topical or systemic antimicrobial drugs (*32*). These factors can result in severe, often polymicrobial, infections of otherwise relatively minor injuries (*32*). Although most documented disaster-associated soft tissue infections are bacterial (typically gram-negative pathogens such as *Aeromonas*, *Escherichia coli*, and *Klebsiella*) (*33*), fungal wound infections can also occur, and they could be under-recognized because they can be clinically similar to bacterial infections, particularly during the early stages of infection.

Mucormycosis, caused by fungi that belong to the order Mucorales, is perhaps the most recognized example of post-disaster fungal soft tissue infection. Necrotizing fasciitis can result, and case-fatality rates of ≈30% are frequently described, although early diagnosis and treatment has been shown to lead to better outcomes (*34*). The first documented instance of disaster-associated mucormycosis occurred after the 1985 volcanic eruption in Armero, Colombia, which caused an estimated 23,000 deaths and ≈4,500 injuries (*17*). According to a report of 38 patients with necrotizing lesions who were hospitalized after the volcano, 8 patients had infections caused by the mucormycete *Rhizopus arrhizus* (*oryzae*) (*17*).

Similarly, a cluster of mucormycosis cases caused by *Apophysomyces trapeziformis* occurred among 13 persons who were severely injured in the May 22, 2011, tornado in Joplin, Missouri, United States (*4*). Penetrating trauma and an increased number of wounds were shown to be independent risk factors for mucormycosis. Whole-genome sequencing of *A. trapeziformis* isolates from case-patients' wounds (Figure) showed 4 nonidentical but closely related strains of *A. trapeziformis*. This finding, considered with case-patients' receipt of medical care at different hospitals, suggested that the infections were acquired from the natural environment as a result of exposure to organic matter and water, which are likely reservoirs for mucormycetes (*4*).

In addition to the 2 clusters described, several isolated cases of post-disaster soft tissue mucormycosis have been reported, notably among persons injured

during the 2004 Indian Ocean tsunami (*13–15*). These reports illustrate some of the clinical challenges associated with soft tissue mucormycosis, caused by organisms that may initially appear indistinguishable from other types of wound infections but require aggressive treatment with intravenous antifungal medication and surgical debridement (*13,14*).

Other agents of fungal soft tissue infections in survivors injured during the 2004 Indian Ocean tsunami include *Fusarium* (which later caused systemic infection) in 1 tourist (*15*) and *Cladophialophora bantiana* in 2 other tourists (*10*). Subcutaneous *C. bantiana* infection has also been associated with a tornado-associated injury in which the patient was inoculated by a contaminated wood splinter but did not have symptoms until ≈16 years later (*35*). Soft tissue fungal infections have also been documented among persons who were not directly injured during a disaster but who sustained minor trauma while performing post-disaster tasks: in Texas after Hurricane Ike in 2008, chromoblastomycosis was diagnosed in 3 patients, all of whom had histories of cancer and all of whom described clearing brush and fallen trees near their homes after the storm (*8*).

### Health Care–associated Fungal Infections after Disasters

Although respiratory and cutaneous infections are the most commonly described forms of fungal infection after natural disasters, other, more invasive fungal infections have also been observed. An outbreak of *Aspergillus* meningitis after the 2004 Indian Ocean tsunami was associated with the use of spinal anesthesia for cesarean section infant delivery for 6 previously healthy women in Sri Lanka (*12*). The first 5 case-patients were initially treated for bacterial meningitis, but the discovery of *Aspergillus* during the post-mortem examination of the index case-patient led to the use of amphotericin B and voriconazole in the surviving case-patients (*12*). Investigation of various medical supplies revealed that syringes from a central storage facility were contaminated with *A. fumigatus*, probably as a result of suboptimal storage conditions in a humid warehouse (*12*).

### Indoor Mold Exposures after Disasters

Disaster-induced water damage to structures can create moist environments that can promote indoor fungal growth, but the extent to which damp indoor spaces and mold growth affect human health remains somewhat ambiguous (*36*). A 2004 report by the Institute of Medicine found sufficient evidence of association between indoor mold exposure and upper respiratory tract symptoms, cough, and wheezing, and evidence of an association between indoor mold exposure and some noninfectious health conditions that included asthma symptoms in persons with asthma and hypersensitivity pneumonitis in some groups of people (*36*). Although the report found no association between indoor mold exposure and invasive infection in healthy persons, there was evidence to support a link between exposure to *Aspergillus* and aspergillosis in severely immunocompromised persons (*36*).

Few data clearly demonstrate that indoor mold exposures increase the risk for invasive infection in post-disaster settings. Flooding lasted for weeks after Hurricanes Katrina and Rita made landfall on the US Gulf Coast in August and September 2005, respectively, leading to visible mold growth in 46% of 112 inspected homes (*37*). Despite these high levels of indoor mold growth documented in some areas, 1 study showed no elevated risk for fungal infections among immunocompromised patients exposed to water-damaged buildings after Hurricane Katrina; 1 patient, 1.2% of the profoundly immunocompromised study population, had a mold infection (caused by a *Cladosporium* sp.), which resolved without antifungal treatment (*9*). Colonization in the absence of related clinical symptoms was observed in persons who returned to their water-damaged homes after Hurricanes Rita and Katrina: the mucormycete *Syncephalastrum* was detected in various clinical specimens from 8 persons whose self-reported exposures to mold ranged from none to heavy, but none had evidence of invasive infection (*38*).

After the 2011 Great East Japan Earthquake and subsequent tsunami, a medical relief team observed unexplained chronic cough among a group of previously healthy persons living in a temporary refuge (*25*). Fungal cultures of sputum samples from 6 persons yielded *Aspergillus fumigatus*, *A. flavus*, and basidiomycetous fungi; culture plates exposed inside the refuge showed a similar fungal profile, suggesting that the indoor environment may have played a role in the patients' infections (*25*). Although the authors of that report state that the patients' coughs resolved after treatment with the antifungal itraconazole, the extent to which an infectious process was responsible for the illnesses is unclear (*25*).

### Disasters, Fungi, and Global Climate Change

Climate change could be affecting the ecology of pathogenic fungi in ways that are not yet fully understood; even minor or gradual changes in temperature, moisture, and wind patterns might affect fungal growth, distribution, and dispersal (*20*). For example, warmer average global temperatures may allow the geographic range of fungi typically restricted to tropical and subtropical environments, such as *Cryptococcus gattii*, to expand into areas that are currently more temperate (*20*). Global warming has also been hypothesized to select for fungi with tolerance to warmer temperatures (*20*). The relative scarcity of fungal diseases among mammals has been hypothesized to

be associated with the inability of many fungal species to survive at temperatures >37°C; however, warmer ambient temperatures may enable nonpathogenic fungi to acquire the ability to infect warm-blooded hosts (*20*).

Huppert and Sparks suggest that global climate change is contributing to greater frequency and severity of extreme weather events and that current patterns of population growth, urbanization, and human activity create conditions that render many communities increasingly vulnerable to these hazards (*39*). Coupled with an increased risk for natural disasters, a larger or more geographically widespread ecologic burden of pathogenic fungi could lead to greater numbers of disaster-associated fungal infections through any of several mechanisms: inhalation of spores dispersed as a result of geophysical disruption, traumatic implantation of fungi into wounds contaminated with organic matter, or infection associated with suboptimal medical care where the local health care system has been damaged or destroyed. Altogether, a combination of factors including genetic and biological aspects of host–pathogen interactions; changing features of the physical environment; and social, political, or economic influences could lead to the emergence of new fungal pathogens or increased numbers of infections by known pathogens (*40*).

## Conclusions

Disasters are complex events that can result in a wide range of health effects, although infectious disease outbreaks as an immediate consequence of disasters are uncommon. Health care providers should be aware of the possibility for cases or clusters of community-acquired or health care–associated fungal infections among disaster survivors because these infections often appear clinically similar to bacterial infections and can be associated with serious illness and death. These infections can occur in persons who do not have the typical immunocompromising risk factors for fungal infection but who have experienced near-drowning, trauma, or other unusual exposure to the environment, such as a dust storm. A fungal infection should be considered early if a patient has a persistent or progressive infection that is not responding to initial antibacterial treatment, particularly because rapid diagnosis and administration of appropriate antifungal therapy can improve patient outcomes. Prompt restoration of disaster-affected aspects of the local health care infrastructure may help facilitate earlier diagnosis and treatment and possibly reduce the risk for infection associated with the use of contaminated medical equipment or substandard care. Strategies to reduce disaster-associated fungal infections should be considered within the broader context of comprehensive and sustainable risk reduction methods to prevent disaster-related injury and illness.

Ms Benedict is an epidemiologist in the Mycotic Diseases Branch, Centers for Disease Control and Prevention. Her interests include the epidemiology of fungal infections and health communications.

Dr Park is a medical officer in the Mycotic Diseases Branch, Centers for Disease Control and Prevention, and leader of the branch epidemiology team. His research interests include the prevention and epidemiology of fungal infections.

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# Monitoring Water Sources for Environmental Reservoirs of Toxigenic *Vibrio cholerae* O1, Haiti

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An epidemic of cholera infections was documented in Haiti for the first time in more than 100 years during October 2010. Cases have continued to occur, raising the question of whether the microorganism has established environmental reservoirs in Haiti. We monitored 14 environmental sites near the towns of Gressier and Leogane during April 2012–March 2013. Toxigenic *Vibrio cholerae* O1 El Tor biotype strains were isolated from 3 (1.7%) of 179 water samples; nontoxigenic O1 *V. cholerae* was isolated from an additional 3 samples. All samples containing *V. cholerae* O1 also contained non-O1 *V. cholerae*. *V. cholerae* O1 was isolated only when water temperatures were ≥31°C. Our data substantiate the presence of toxigenic *V. cholerae* O1 in the aquatic environment in Haiti. These isolations may reflect establishment of long-term environmental reservoirs in Haiti, which may complicate eradication of cholera from this coastal country.

Epidemic cholera was identified during October 2010 in Haiti; initial cases were concentrated along the Artibonite River (*1*,*2*). The clonal nature of isolates during this initial period of the epidemic has been described (*3*–*6*). Because cholera had not been reported in Haiti for at least 100 years, there is a high likelihood that the responsible toxigenic *Vibrio cholerae* strain was introduced into Haiti, possibly through Nepalese peacekeeping troops garrisoned at a camp along the Artibonite River (*4*,*7*). In the months after October 2010, cholera spread quickly through the rest of Haiti: 604,634 cases and 7,436 deaths were reported in the first year of the epidemic (*1*). In the intervening years, cases and epidemics have been reported, and it has been suggested that onset of the rainy season serves as a trigger for disease occurrences (*2*,*8*).

*V. cholerae* is well recognized as an autochthonous aquatic microorganism species with the ability to survive indefinitely in aquatic reservoirs and is possibly in a "persister" phenotype (*9*). *V. cholerae* strains can also persist in aquatic reservoirs as a rugose variant that promotes formation of a biofilm that confers resistance to chlorine and to oxidative and osmotic stresses (*10*–*13*) and also persists in a viable but nonculturable form (*14*). Work by our group and others suggests that cholera epidemics among humans are preceded by an environmental bloom of the microorganism and subsequent spillover into human populations (*15*–*17*). In our studies in Peru (*16*), water temperature was found to be the primary trigger for these environmental blooms and could be correlated with subsequent increases in environmental counts and occurrence of human illness.

To understand patterns of ongoing cholera transmission and seasonality of cholera in Haiti, and to assess the likelihood of future epidemics, it is essential to know whether environmental reservoirs of toxigenic *V. cholerae* O1 have been established, where these reservoirs are located, and what factors affect the occurrence and growth of the microorganism in the environment. We report the results of an initial year of monitoring of environmental sites in the Ouest Department of Haiti, near the towns of Leogane and Gressier, where the University of Florida (Gainesville, FL, USA) has established a research laboratory and field area.

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## Methods

### Environmental Sampling Sites

Fifteen fixed environmental sampling sites were selected near Gressier and Leogane (Figures 1,2). Sites were selected along transects of 3 rivers in the area and at 1 independent estuarine site: the Momance River (4 up-river sites and 1 estuarine site at the mouth of the river), the Gressier River (4 up-river sites and 1 estuarine site at the mouth of the river), the Tapion River (4 river sites), and an independent estuarine site at Four-a-chaux, which is a historic ruin and tourist attraction. All sites were ≥0.5 miles apart, with the exception of the Christianville Bridge and Spring sites, which were 0.25 miles apart. Topography of this area is typical for Haiti: rivers originated in the mountains (peaks in the region are >8,000 feet) and flowed into a broad flood plain where Gressier and Leogane were located. Up-river sites on the Momance and Gressier Rivers were in the

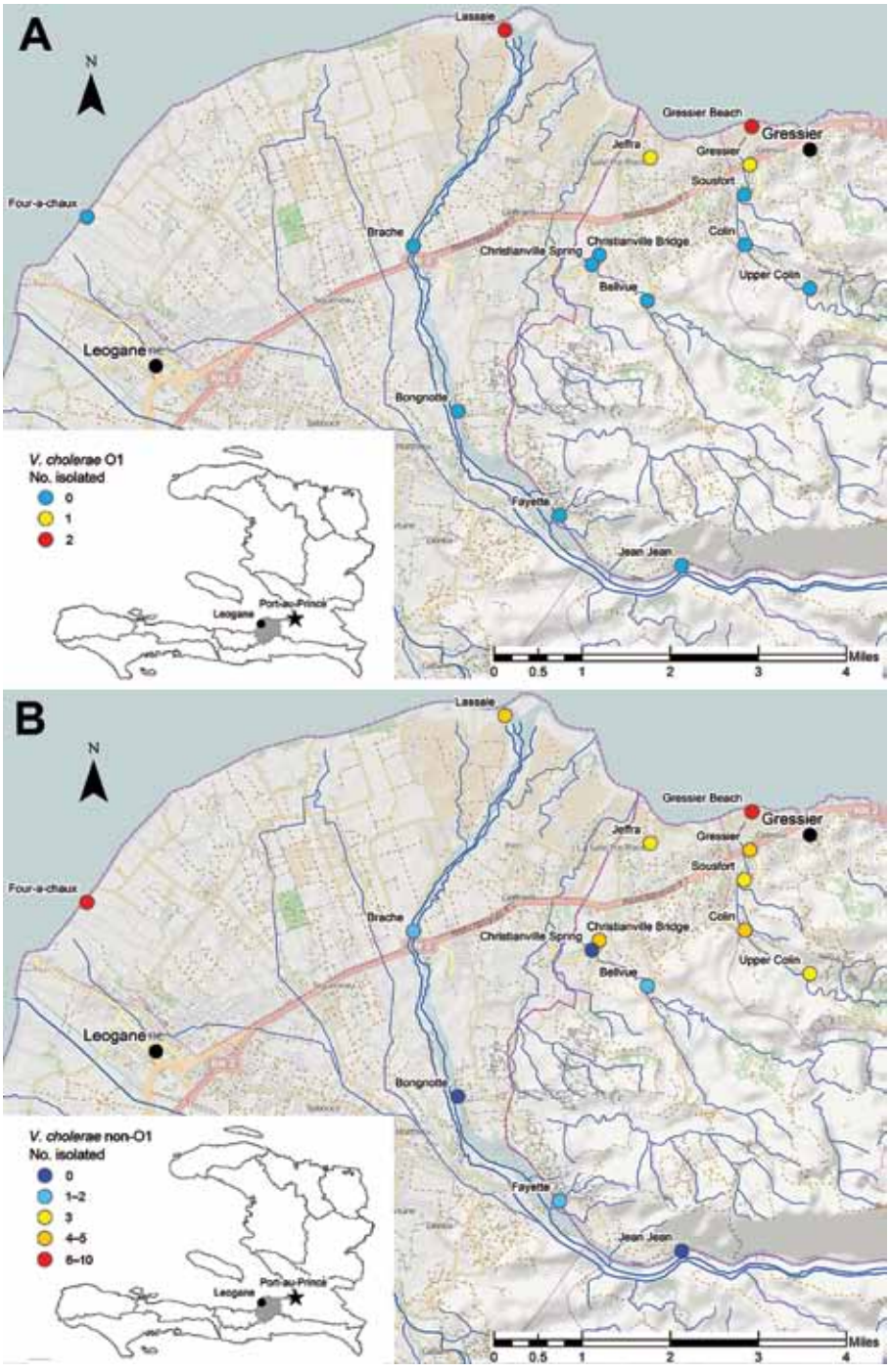


Figure 1. Locations of environmental sampling sites near the towns of Gressier and Leogane in Haiti. Samples were collected during April 2012–March 2013. A) Number of *Vibrio cholerae* O1 isolates obtained from sampling sites. B) Number of non-O1/non-O139 *V. cholerae* isolates obtained from sampling sites. The number of *V. cholerae* isolates obtained from each sampling site is indicated by distinct color coding.

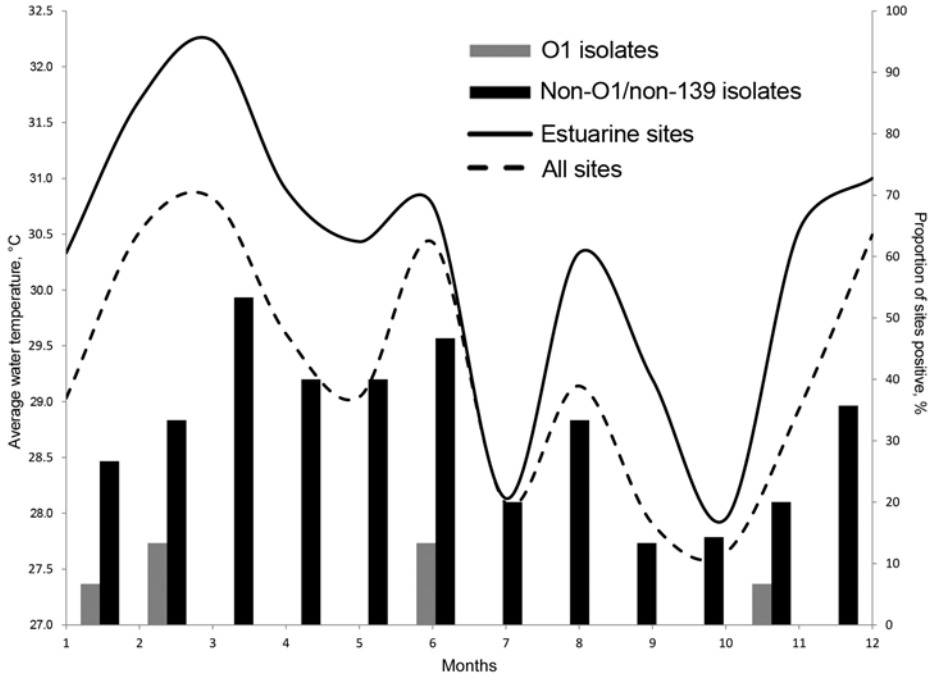


Figure 2. Mean combined water temperature for all sites monitored in the Ouest Department of Haiti, near the towns of Leogane and Gressier, and percentage of environmental sites positive for *Vibrio cholerae* O1 or non-O1/non-O139, by month.

mountains, where human populations are limited. Water samples were collected once a month from each site during April 2012–March 2013. A total of 179 samples were collected for culture for *V. cholerae*; 176 samples were available for measurement of water quality parameters.

### Isolation and Identification of *V. cholerae* from Environmental Sites

For the isolation of *V. cholerae,* 500 ml of water was collected in a sterile 500-mL Nalgene (http://nalgene.com/) bottle from each fixed site; the samples were transported at ambient temperature to the University of Florida laboratory at Gressier and processed for detection of *V. cholerae* within 3 hours of collection.

In addition to the conventional sample enrichment technique (*18*), we used alkaline peptone water (APW) to enrich water samples. A 1.5-mL water sample was enriched with 1.5 mL of 2× APW in 3 tubes: 1 tube was incubated at 37°C for 6–8 hours (*18*), another tube was incubated overnight at 37°C, and the third tube was incubated at 40°C for 6–8 hours. Subsequently, a loopful of culture from each tube was streaked onto thiosulfate citrate bile salts sucrose agar (Becton-Dickinson, Franklin Lakes, NJ, USA), and the plates were incubated overnight at 37°C. From each plate, 6–8 yellow colonies exhibiting diverse morphology were transferred to L-agar; these plates were incubated overnight at 37°C. Each colony was examined by using the oxidase test; oxidase-positive colonies were tested by using *V. cholerae* O1–specific polyvalent antiserum and O139-specific antiserum (DENKA SEIKEN Co., Ltd, Tokyo, Japan). The isolates were further examined by using colony PCR for the presence of *ompW* and *toxR* genes specific for *V. cholerae* spp. as described (*9*).

### Screening of Aquatic Animals and Plants

To determine whether they serve as reservoirs for *V. cholerae* O1, we collected aquatic animals typically eaten by humans, including shrimp, fish, crab, crayfish, and aquatic plants (n = 144) weekly during February 5–22, 2013. The samples were collected from 14 environmental sites. Each sample was placed into a sterile plastic seam–locking bag and transported to the laboratory. One gram of the sample was mixed with 100 mL of saline and then homogenized in a sterile blender; 1.5 ml of the resultant mixture was enriched in 2× APW and processed as described.

### Genetic Characterization of *V. cholerae* O1 Strains

To further characterize the environmental *V. cholerae* O1 serogroup Ogawa biotype El Tor strains, we subjected all *V. cholerae* O1 isolates from water and seafood to PCR analysis for key virulence genes, including *ctxA*, *ctxB*$^{-CL}$, (MAMA$^{-CL}$), *ctxB*-$^{ET}$ (MAMA$^{-ET}$), *rstR*$^{-ET}$, *rstR*$^{-CL}$, *rstC*$^{-ET}$, *rstC*$^{-CL}$, *tcpA*$^{-CL}$, and *tcpA*$^{-ET}$, as described (*19*,*20*). The chromosomal DNA was extracted from each strain by using a GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA), and the DNA was used for PCR templates; the PCR conditions were as described (*3*).

### Aerobic Plate Counts

To determine total aerobic bacterial counts in water samples, we plated undiluted, 10- and 100-fold dilutions of water onto L-agar and incubated overnight at 37°C.

The countable plates (100–300 colonies) were used to determine the total (CFU/mL) culturable bacteria present in the water samples.

### Water Parameters, Rainfall, and Human Case Counts

When collecting water samples, we measured physical parameters, including pH, water temperature, dissolved oxygen, total dissolved solids, salinity, and conductivity in the field sites by using a HACH portable meter (HACH Company, Loveland, CO, USA) and designated electrodes following the manufacturer's recommendations. Rainfall estimates were based on National Aeronautics and Space Administration data for the study region bounded by the rectangle (18.2°–18.6°N, 17.1°–17.8°W) by using the average daily rainfall measurement tool, Tropical Rainfall Measuring Mission 3B42_daily (*21*). Estimates of average precipitation (mm/day) with a spatial resolution of 0.25 × 0.25 degrees were aggregated to obtain weekly accumulated rainfall measurements during the study period. Cholera incidence data were obtained from daily reports by Ouest Department (excluding Port-au-Prince) to the Haitian Ministry of Public Health and Population and aggregated to total cases per week during April 20, 2012–March 27, 2013 (*22*).

### Data Analysis

We examined the effects of water quality factors on the presence of toxigenic and nontoxigenic *V. cholerae* by conditional logistic regression after stratification for the site. Stratification excluded sites that had all-positive or all-negative outcomes; of the remaining sites, regression analysis showed O1 *V. cholerae* in 47 observations from 4 sites and non-O1/non-O139 *V. cholerae* in 154 observations from13 sites. As shown in Figure 1, we performed cartography by using ArcGIS version 10 (ESRI, Redlands, CA, USA).

## Results

*V. cholerae* O1 serogroup Ogawa biotype El Tor was isolated from 6 (3.4%) of the 179 water samples and 1 (0.7%) of the 144 aquatic animal and plant samples by using modified APW enrichment techniques. Of those 7 environmental isolates, 3 (43%) were confirmed as *ctx*-positve toxigenic *V. cholerae* O1 strains, and 4 (57%) were confirmed as *ctx*-negative *V. cholerae* O1 strains by using genetic analysis as described below. As shown in Table 1, APW enrichment at 37°C overnight or incubation at 40°C for 6–8 hours, or both, enhanced the rate of isolation of *V. cholerae* O1 from samples. PCR analysis of the key virulence genes showed that 3 (43%) of the 7 isolates, all from water, were positive for key virulence genes, including cholera toxin genes and *tcpA* genes, and that 4 (57%) isolates exhibited no cholera toxin bacteriophage (CTXΦ)–related genes (*23*; Table 2). To further assess the PCR results, we sequenced DNA flanking the CTXΦ from 1 strain, Env-9 (Table 2). Sequence data corroborated PCR results that indicated that Env-9 lacked CTXΦ.

Physical parameters for the environmental water samples are summarized in Table 3. Because the sites varied from mountains to floodplain to estuaries, there was relatively wide variability in salinity (0–21.6 g/L), pH (6.4–8.6), and temperature (24.3–33.7°C). Temperatures tended to increase as rivers approached the sea. As shown in Figure 2, mean water temperature from all sites showed evidence of seasonal variation. Measurement of rainfall was available for the region as a whole (Figure 3). However, site-specific rainfall data were not available; consequently, rainfall was not included in the regression models.

Isolation of *V. cholerae* O1 strains was most common from the sites at the mouths of the Momance and Gressier Rivers (Figure 1, panel A). In a conditional logistic regression analysis with water quality factors (Table 4), the only variable that emerged as statistically significant was water temperature (odds ratio 2.14, 95% CI 1.06–4.31); all isolations of *V. cholerae* O1 (toxigenic and nontoxigenic) occurred at water temperatures of ≥31°C. As shown in Figure 3, there was evidence that *V. cholerae* O1 isolation was more common in the environment preceding epidemic peaks of disease among humans; however, numbers of isolations were too small to permit statistical analysis. Of 179 samples, the only *V. cholerae* O1 isolate from aquatic animals or plants was from a shrimp sample and was nontoxigenic; it was collected simultaneously with a water sample that was also positive for nontoxigenic *V. cholerae* O1.

Non-O1 *V. cholerae* was much more common in the environment than *V. cholerae* O1 strains and was isolated from 56 (31%) of 179 water samples. As observed with O1 strains, isolations were more common at the mouths of the rivers and in estuarine areas (Figure 1, panel B); however, the non-O1 strain was found farther upriver than were O1 strains and was isolated from several sites in the mountains. Non-O1 strains were isolated from all sites that were also positive for O1 strains. Non-O1 strains were isolated in all months, without an obvious association with regional

Table 1. Effect of diverse enrichment conditions on the isolation of culturable *Vibrio cholerae* O1 strains from aquatic reservoirs in the Gressier and Leogane regions of Haiti

| | Culture results after alkaline peptone water enrichment | | |
|---|---|---|---|
| Strain ID | 37°C (6–8 h) | 37°C (18–24 h) | 40°C (6–8 h) |
| Env-9 | - | - | + |
| Env-90 | - | - | + |
| Env-94 | + | - | + |
| Env-122* | + | - | - |
| Env-383 | - | + | - |
| Env-390 | + | - | - |
| Env-114* | - | + | + |

*Env-122 and env-114 were isolated from water and a shrimp sample, respectively, from a single sampling site at a single isolation round.

Table 2. PCR analysis of genes of *ctx* –positive toxigenic *Vibrio cholerae* O1 strains and *ctx*-negative *V. cholerae* O1 strain

| Strain | PCR | | | | | | | | | | Mismatch amplification mutation assay PCR | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *ompW* | *toxR* | *tcpA*$^{CL}$ | *tcpA*$^{ET}$ | *ctxA* | *ctxB* | *rstR*$^{ET}$ | *rstR*$^{CL}$ | *rstC*$^{ET}$ | *rstC*$^{CL}$ | *ctxB*$^{CL}$ | *ctxB*$^{ET}$ |
| Env-9* | + | + | + | - | - | - | - | - | - | - | - | - |
| Env-90 | + | + | - | + | + | + | + | - | - | - | + | - |
| Env-94 | + | + | - | + | + | + | + | - | - | - | + | - |
| Env-122*† | + | + | + | - | - | - | - | - | - | - | - | - |
| Env-383 | + | + | - | + | + | + | + | - | - | - | + | - |
| Env-390* | + | + | + | - | - | - | - | - | - | - | - | - |
| Env-114*† | + | + | + | - | - | - | - | - | - | - | - | - |

*Indicates *ctx*-negative *V. cholerae* O1 isolates.
†Env-122 and env-114 were isolated from water and a shrimp sample, respectively, from a single sampling site and at a single isolation round.

rainfall totals or cholera incidence. In a conditional logistic regression analysis, isolation of non-O1 strains was significantly associated ($p<0.05$) with higher water temperature and moderate levels of dissolved oxygen (Table 4).

## Discussion

Before this study, isolation of 2 toxigenic *V. cholerae* O1 strains from large-volume water samples (30 L) was reported in the Artibonite region (*24*); other studies at that time suggested that *V. cholerae* O1 strains were not present, or present at only minimal levels (*2*,*25*) in the environment in Haiti. In contrast, we isolated *ctx*-positive and *ctx*-negative *V. cholerae* O1 serogroup Ogawa biotype El Tor strains (Table 1) in the environment at a frequency comparable to that reported from cholera-endemic areas such as Bangladesh (*17*). Our successful isolation of the microorganism from the environment may reflect localization of environmental isolates near Gressier and Leogane, where our study was conducted; however, we believe that our findings are more likely to be a reflection of the method used. Data presented here suggest that, in addition to conventional APW enrichment, longer APW enrichment time and enrichment at higher temperatures contributed to an increased rate of isolation of *V. cholerae* O1 strains from aquatic environmental reservoirs (Table 1), resulting in successful isolation from 1.5-mL water samples. We also note some issues relating to sample transport: Baron et al. (*25*) transported their water samples on ice in coolers; our samples were transported at room temperature. As has been reported, *Vibrio* spp. are extremely sensitive to low temperatures (*26*), and in our experience, transport of samples on ice resulted in a marked reduction in isolation rates.

Water from which we isolated *V. cholerae* spp. tended to have been sampled at the point where rivers meet the sea, and in adjacent estuarine areas, again following the patterns reported from Bangladesh (*17*). Water temperature was found to be the single physical parameter that was substantially associated with isolation of these organisms; higher temperatures were concentrated downriver and in estuarine areas. For our analysis, we used a conditional logistic regression model to permit stratification by site. Although we found very low numbers for *V. cholerae* O1 isolates (6 positive water samples), results coincided with the non-O1 results and the exploratory data analysis. In our studies of aquatic animals likely to be eaten by humans, we did isolate *V. cholerae* O1 from shrimp in 1 instance. The isolate was nontoxigenic; consequently, its association with disease is unclear.

After analyzing the results of this study, we asked the following question: has *V. cholerae* O1 become established in environmental reservoirs in Haiti? Toxigenic *V. cholerae* O1 strains are clearly present in the environment, and it may be that the isolates that we identified are the result of fecal contamination of the environment by persons infected with *V. cholera* strains. Although data are limited, there was at least a suggestion that isolation of *V. cholera* strains from environmental reservoirs was more common at the beginning of epidemic spikes of human disease (as has been described in association with environmental reservoirs) (*16*,*17*,*27*) rather than at the height of epidemics among humans, as might have been

Table 3. Summary statistics of environmental water quality factors in mountains, estuaries, and a floodplain in Haiti, April 2012–March 2013

| Water quality | No. specimens observed | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|
| Temperature, °C | 176 | 29.32 | 1.91 | 24.3 | 33.7 |
| pH, log[H+] | 176 | 7.71 | 0.36 | 6.4 | 8.6 |
| Dissolved oxygen, mg/L | 176 | 7.33 | 1.81 | 1.23 | 10.31 |
| Total dissolved solids, mg/L | 176 | 273.8 | 359.9 | 24 | 2,970 |
| Salinity, g/L | 176 | 0.38 | 1.72 | 0 | 21.6 |
| Conductivity (µS/cm) | 176 | 544.1 | 639.4 | 230 | 5,630 |
| Heterotrophic bacteria, log(cfu/mL) | 175 | 4.21 | 0.60 | 2.3 | 5.89 |

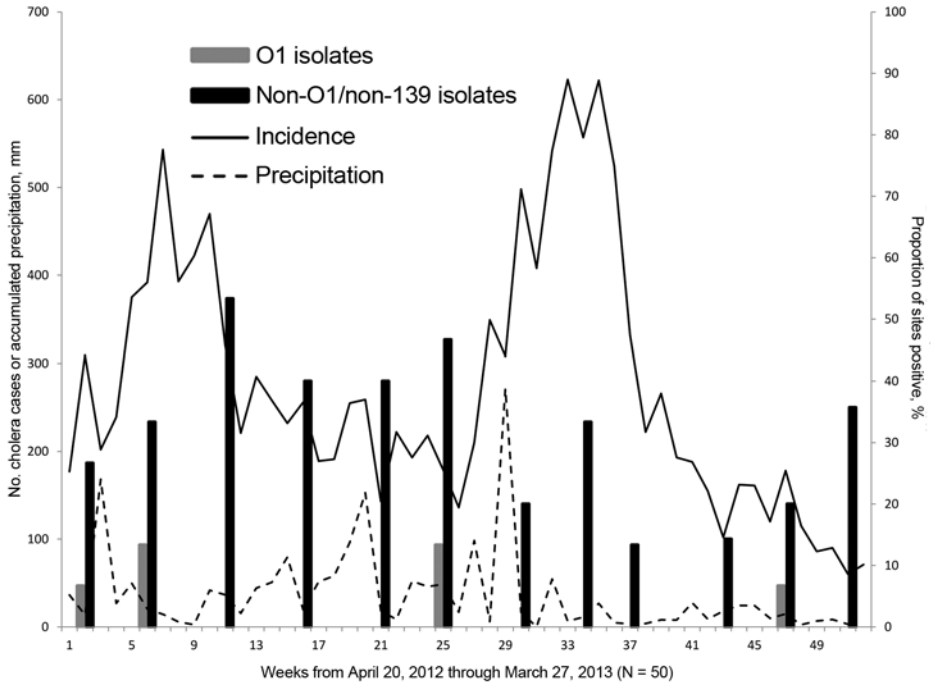


Figure 3. Weekly cholera case incidence for Ouest Department, excluding Port-au-Prince, Haiti, based on data reported to the Haitian Ministry of Public Health and Population and regional precipitation by week during April 2012–March 2013, combined with percentage of environmental sites from which *V. cholerae* O1 or non-O1/non-O139 were isolated, by month.

expected related to fecal contamination. We also found non-O1 strains widely distributed throughout the environment, including mountain river sites, consistent with widespread dissemination in environmental reservoirs. Although we cannot be certain that O1 and non-O1 strains grow under comparable conditions, the clear establishment of non-O1 *V. cholerae* strains in environmental reservoirs suggests that conditions are appropriate for growth of *V. cholerae* O1 strains. Of potentially greater interest is the observation that only 3 of the 7 (47%) *V. cholerae* O1 biotype El Tor strains isolated carried the *ctx* genes (Table 2). Data from 1 *ctx*-negative strain (Env-9) was consistent with absence of the entire CTXΦ. We propose that the 3 isolates that are positive for *ctx* genes be classified as circulating *V. cholerae* altered biotype El Tor strains in Haiti. To better understand the evolutionary mechanisms involved, we are performing further sequence analysis of clinical and environmental strains.

## Conclusions

The apparent introduction of toxigenic *V. cholerae* O1 in Haiti in 2010, after decades during which no cholera cases were reported, was unquestionably a public health disaster. If these O1 strains establish stable environmental reservoirs in Haiti, in the setting of ongoing problems with water and sanitation, there is a high likelihood that we will see recurrent epidemics

Table 4. Conditional logistic regression analysis of water quality factors affecting the occurrence *Vibrio cholerae* O1 and non-O1/non-O139 in aquatic reservoirs, Haiti, April 2012–March 2013

| Factor | Units | No. observations | Odds ratio (95% CI) | p value |
|---|---|---|---|---|
| Presence of *V. cholerae* O1 | | | | |
| Temperature | 1°C | 47 | 2.14 (1.06–4.31) | 0.033* |
| pH | 1 log[H+] | 47 | 0.01 (0.00–1.81) | 0.083 |
| Dissolved oxygen | 1 mg/L | 47 | 0.32 (0.08–1.20) | 0.091 |
| Total dissolved solids | 100 mg/L | 47 | 1.08 (0.95–1.23) | 0.258 |
| Salinity | 1 g/L | 47 | 1.24 (0.86–1.80) | 0.254 |
| Conductivity | 100 (μS/cm) | 47 | 1.05 (0.98–1.13) | 0.198 |
| Heterotrophic bacteria | log (CFU/mL) | 47 | 6.00 (0.57–62.78) | 0.135 |
| Presence of *V. cholerae* non-O1 | | | | |
| Temperature | 1°C | 154 | 1.36 (1.05–1.76) | 0.02* |
| pH | 1 log[H+] | 154 | 0.44 (0.09–2.14) | 0.311 |
| Dissolved oxygen | 1 mg/L | 154 | 0.50 (0.32–0.79) | 0.003* |
| Total dissolved solids | 100 mg/L | 154 | 0.96 (0.86–1.06) | 0.413 |
| Salinity | 1 g/L | 154 | 1.19 (0.80–1.77) | 0.378 |
| Conductivity | 100 (μS/cm) | 154 | 0.98 (0.92–1.04) | 0.432 |
| Heterotrophic bacteria | log (CFU/mL) | 153 | 2.35 (0.95–5.77) | 0.063 |

*p<0.05 were considered statistically significant.

within the country. These circumstances clearly have implications for current plans by the Haitian Ministry of Public Health to eradicate cholera in Haiti within a decade (*28*). The proposed implementation of vaccination programs and efforts to improve water supplies and sanitation will undoubtedly reduce case numbers, but as long as the causative microorganism is present in the environment, eradication of the disease will not be possible. Establishment of environmental reservoirs and recurrent epidemics may also serve as a potential source for transmission of the disease to the Dominican Republic and other parts of the Caribbean (*1*). Ongoing monitoring of potential environmental reservoirs in the areas near Gressier and Leogane as well as in sentinel sites throughout the country will be necessary to assess this risk and to permit development of rational public health interventions for cholera control.

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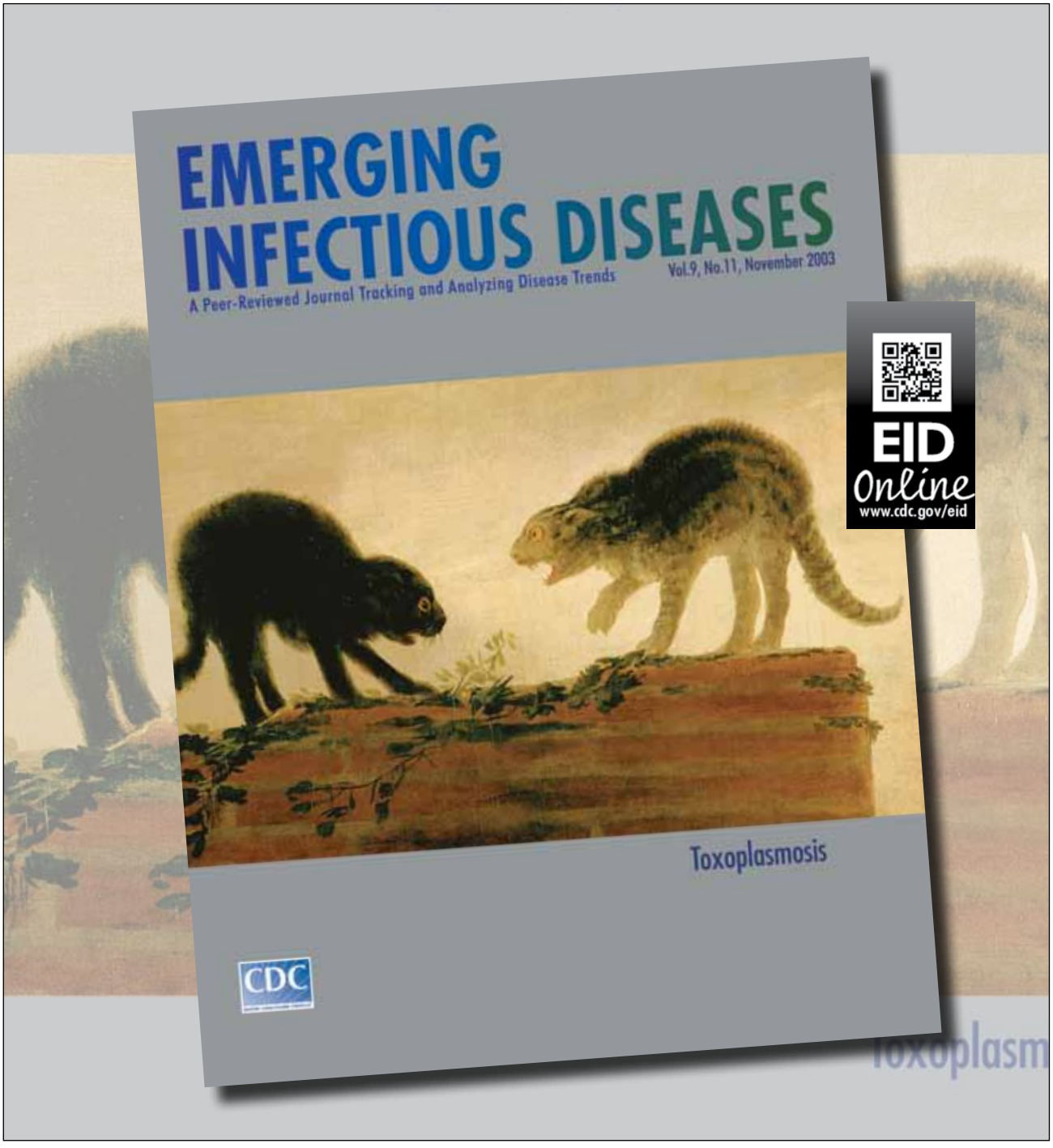

# High-level Relatedness among *Mycobacterium abscessus* subsp. *massiliense* Strains from Widely Separated Outbreaks

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Three recently sequenced strains isolated from patients during an outbreak of *Mycobacterium abscessus* subsp. *massiliense* infections at a cystic fibrosis center in the United States were compared with 6 strains from an outbreak at a cystic fibrosis center in the United Kingdom and worldwide strains. Strains from the 2 cystic fibrosis outbreaks showed high-level relatedness with each other and major-level relatedness with strains that caused soft tissue infections during an epidemic in Brazil. We identified unique single-nucleotide polymorphisms in cystic fibrosis and soft tissue outbreak strains, separate single-nucleotide polymorphisms only in cystic fibrosis outbreak strains, and unique genomic traits for each subset of isolates. Our findings highlight the necessity of identifying *M. abscessus* to the subspecies level and screening all cystic fibrosis isolates for relatedness to these outbreak strains. We propose 2 diagnostic strategies that use partial sequencing of *rpoB* and *secA1* genes and a multilocus sequence typing protocol.

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Nontuberculous mycobacteria (NTM) and, in particular, the *Mycobacterium abscessus* group are recognized as emerging respiratory pathogens among patients with cystic fibrosis. Reports from the United States, France, and Israel have shown that the *M. abscessus* group accounts for a major proportion of NTM infections in patients with cystic fibrosis; prevalence rates range from 16% to 48% (*1*–*3*).

Previous studies have indicated great diversity within *M. abscessus* group strains among cystic fibrosis patients, suggesting independent acquisitions of NTM from the environment (*2*,*4*). However, suspicion of patient-to-patient transmission arose with the recent report of an outbreak of respiratory infection with *M. abscessus* subsp. *massiliense* at a cystic fibrosis center in Seattle, Washington, USA (*5*). The index case-patient and 4 additional patients all had multidrug-resistant isolates with resistance to amikacin and clarithromycin. All 5 strains were indistinguishable by repetitive unit sequence–based PCR patterns and pulsed-field gel electrophoresis analysis, which led to initiation of whole-genome sequencing. In a separate, recent study, whole-genome sequencing and epidemiologic analysis provided strong support for patient-to-patient transmission in 2 clustered outbreaks of *M. abscessus* subsp. *massiliense* at the Papworth Hospital Cystic Fibrosis Centre (Cambridge, UK) (*6*). Isolates from both clusters showed resistance to clarithromycin, and isolates from one of the clusters also had mutations conferring resistance to amikacin.

The availability of whole-genome sequences from different *M. abscessus* subsp. *massiliense* outbreaks, as well as unrelated strains, provides an unprecedented opportunity for multigenome comparisons. We conducted a genomic study of 3 recently sequenced strains from the Seattle cystic

fibrosis outbreak, including the index strain, and compared them with representative strains from the Papworth cystic fibrosis outbreak, as well as with available strains from the United Kingdom, the United States, Brazil, South Korea, France, and Malaysia (Table 1). We found high-level relatedness among strains from the 2 geographically distant outbreaks in Seattle and Papworth. We also identified shared and unique genomic traits for strains from both cystic fibrosis outbreaks and for those from an outbreak of soft tissue infections in Brazil.

## Materials and Methods

### Sequence Analysis of Outbreak Strains

A subset of 6 isolates (2u, 12c, 14h, 19f, 20h, and 28c) representing the breadth of genomic diversity observed within the Papworth cystic fibrosis outbreak clusters 1 and 2 (*6*) were selected. Illumina sequencing reads from each of these isolates were assembled into sets of contigs by using Velvet software (*21*). These contigs were combined with draft genome sequences of the Seattle cystic fibrosis outbreak and available whole-genome sequences of *M. abscessus* subsp. *massiliense* (Table 1) and subjected to whole-genome multiple sequence alignments by using Mugsy software (*22*). Core segments of the alignment that are shared among all isolates included in the analysis were identified and concatenated by using Phylomark software (*23*). Concatenated nucleotide sequences, including single-nucleotide polymorphisms (SNPs), were then used for construction of a neighbor-joining phylogenetic tree by using MEGA software (*24*). The use of microbial samples and data was approved by the ethics committees at each of the institutions involved.

To replicate data from the Papworth cystic fibrosis outbreak clusters 1 and 2 (*6*) by using a similar approach, we mapped sequencing reads from the subset of 6 Papworth isolates, together with reads with from the 3 Seattle cystic fibrosis isolates and soft tissue strain CRM-0020 from Brazil (Table 1), onto the *M. abscessus* type strain ATCC 19977$^T$ reference genome by using BWA software (*25*). Variants, including SNPs, were called by using GATK software (*26*) and filtered for quality. The SNP panel was used for construction of a neighbor-joining phylogenetic tree by using MEGA software. The resulting tree replicated the topology of clusters 1 and 2 and showed that the Seattle isolates are most closely related to cluster 2.

### PCR and In Silico PCR

Standard PCR and sequencing strategies were used to amplify and analyze partial sequences of the *rpoB* (723 bp) (*27,28*) and *secA1* (465 bp) (*29*) genes. In addition, a multilocus sequence typing (MLST) scheme (*29,30*), including primers to 13 housekeeping genes (*cya*, *gdhA*, *argH*, *glpK*, *gnd*, *murC*, *pgm*, *pknA*, *pta*, *pur*, *rpoB*, *hsp65*, and *secA1*) was used to conduct electronic PCR on the panel of 20 *M. abscessus* subsp. *massiliense* genomes (Table 1). Published forward primers for *cya* and *gdhA* (*30*) did not amplify in silico for some *M. abscessus* subsp. *massiliense* strains; therefore, the following new primers conserved across the *M. abscessus* group were used: cya_F_new 5′-GCC TGC GTA AGG GTG ATG-3′ and gdhA_F_new 5′-GTG AAG CTC GCC GCC TGC-3′. Alleles from each gene were

Table 1. Twenty-four *Mycobacterium abscessus* group strain genomes analyzed for genetic relatedness*

| Subspecies/strain | Country | Outbreak | GenBank accession no. | Reference |
|---|---|---|---|---|
| Mm/2u | UK | Papworth | NA | (*6*) |
| Mm/12c | UK | Papworth | NA | (*6*) |
| Mm/14h | UK | Papworth | NA | (*6*) |
| Mm/19f | UK | Papworth | NA | (*6*) |
| Mm/20h | UK | Papworth | NA | (*6*) |
| Mm/28c | UK | Papworth | NA | (*6*) |
| Mm/2B-0107 | USA | Seattle | AKUN00000000 | This study |
| Mm/MAB_082312_2258 | USA | Seattle | AYTA00000000 | This study |
| Mm/MAB_091912_2446 | USA | Seattle | AYTF00000000 | This study |
| Mm/CRM-0020 | Brazil | Rio de Janeiro | ATFQ00000000 | (*7*) |
| Mm/GO-06 | Brazil | Goiás | CP003699 | (*8*) |
| Mm/47J26 | UK | Not applicable | AGQU01000000 | (*9*) |
| Mm/M18 | Malaysia | Not applicable | AJSC01000000 | (*10*) |
| Mm/M115 | Malaysia | Not applicable | AJLZ00000000 | (*11*) |
| Mm/M139 | Malaysia | Not applicable | AKVR01000000 | (*12*) |
| Mm/M154 | Malaysia | Not applicable | AJMA01000000 | (*13*) |
| Mm/Asan 50594 | South Korea | Not applicable | CP004374–CP004376 | (*14*) |
| Mm/1S-151–930 | USA | Not applicable | AKUI00000000 | This study |
| Mm/5S-0817 | USA | Not applicable | AKUB00000000 | This study |
| Mm/CCUG 48898$^T$ | France | Not applicable | AKVF01000000 | (*15,16*) |
| Ma/CF | France | Not applicable | CAHZ00000000 | (*17*) |
| Ma/ATCC 19977$^T$ | USA | Not applicable | CU458896,CU458745 | (*18*) |
| Mb/BD$^T$ | France | Not applicable | AHAS00000000 | (*19*) |
| Mb/M24 | Malaysia | Not applicable | AJLY00000000 | (*20*) |

*Mm, *M. abscessus* subsp. *massiliense*; NA, not available; Ma, *M. abscessus* subsp. *abscessus*; Mb, *M. abscessus* subsp. *bolletii*.

extracted and concatenated for each genome, the panel of concatenated sequences was aligned by using ClustalW software (*31*), and the core segments of the alignment were used for construction of a neighbor-joining phylogenetic tree by using MEGA software.

## Results

### Phylogenetic Characteristics of Outbreak Strains

A core genome phylogenetic tree (Figure 1) showed a tight cluster of the 3 Seattle cystic fibrosis outbreak strains. The Seattle cystic fibrosis cluster was closely related to the 2 cystic fibrosis clusters described for the Papworth outbreak (*6*) and the Birmingham, UK, cystic fibrosis isolate 47J26 (*9*). Furthermore, the Seattle and Papworth cystic fibrosis outbreak strains showed some relatedness to strains CRM-0020 and GO-06 derived strains (known collectively as BRA-100) isolated during an epidemic of soft tissue infections in Brazil (*32*) and the *M. abscessus* subsp. *massiliense* M18 strain from Malaysia (*10*).

The cumulative size of core segments of Mugsy alignments provides information on relatedness among groups of strains compared. The core genome reduces in size as more genomes are added; an expected major decrease occurs after addition of more distant strains to the group. The average genome size of cystic fibrosis outbreak strains was 4.81 Mb for Seattle (n = 3) and 4.97 Mb for Papworth (n = 6). The Seattle and Papworth cystic fibrosis outbreak strains (n = 9) shared a core genome of 4,264,844 nt, which is almost unchanged by including the Birmingham cystic fibrosis strain 47J26 (n = 10; 4,264,127 nt). Addition of the soft tissue outbreak strain CRM-0020 from Brazil (n = 11) (*32*) decreased the core to 4,231,390 nt, and adding the related outbreak strain GO 06 from Brazil (n = 12) (*8,33*), led to an additional decrease in the core genome to 4,043,718 nt. As expected, including unrelated available clinical *M. abscessus* subsp. *massiliense* strains (n = 20, including M139 with ambiguous subspecies taxonomic assignment (*12*), (Table 1), reduced the core genome size to 3,869,950 nt. Further addition of *M. abscessus* subsp. *abscessus* (n = 2) and *M. abscessus* subsp. *bolletii* (n = 2) genomes (Table 1) reduced the core to 3,828,656 nt.

### Genomes of Strains from Cystic Fibrosis and Soft Tissue Infection Outbreaks

The core genome of 10 strains representing the Papworth cystic fibrosis (n = 6), the Seattle cystic fibrosis (n = 3), and soft tissue CRM-0020 (n = 1) outbreaks comprised 4,231,390 nt. Strain GO 06 was excluded from the analysis because its genome harbors a large number of ambiguous nucleotides and an unusual hybrid appearance with fragments of *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *abscessus* sequences. Strains 47J26 and M18, isolated from the sputum of a cystic fibrosis patient in Birmingham, UK, and a lymph node sample from a patient in Malaysia, respectively, were related to the outbreak strains

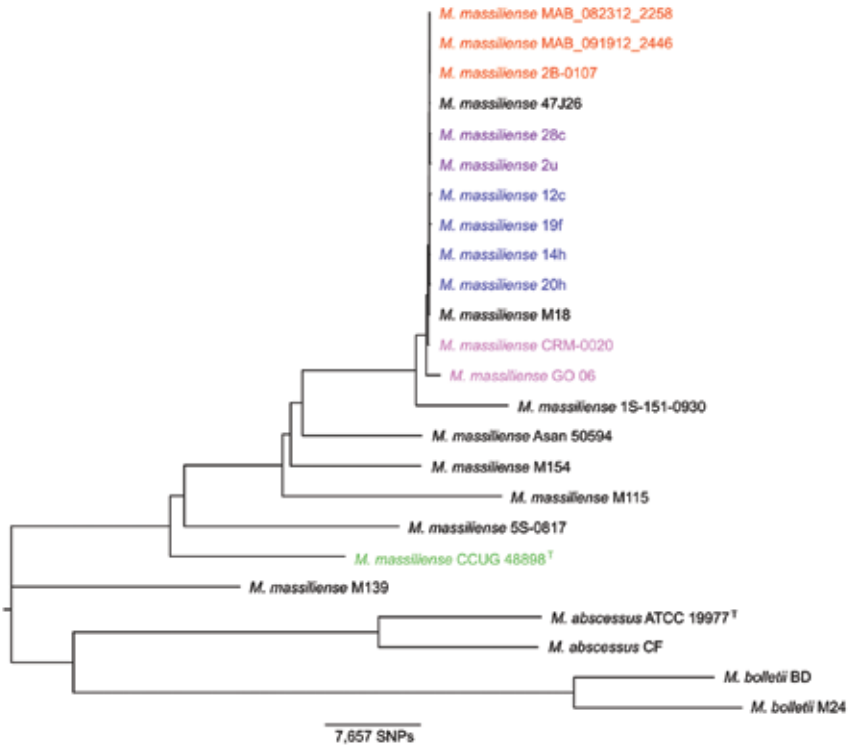


Figure 1. Neighbor-joining phylogenetic tree based on whole-genome multiple alignment of 24 *Mycobacterium abscessus* group genomes. Genomes in Table 1 were aligned by using Mugsy (*22*), core segments of the alignment were identified by using Phylomark (*23*), and resulting concatenated nucleotide sequences were used for construction of the midpoint-rooted neighbor-joining phylogenetic tree by using MEGA (*24*). Strains from an outbreak of *M. abscessus* subsp. *massiliense* infections at a cystic fibrosis center in Seattle, Washington, USA, are indicated in red; strains from an outbreak of *M. abscessus* subsp. *massiliense* infections at a cystic fibrosis center in Papworth, UK, are indicated in blue (cluster 1) and purple (cluster 2); strains from Brazil are indicated in magenta; and the *M. abscessus* subsp. *massiliense* type strain is indicated in green. Boostrap values obtained after 100 iterations were ≥97 for all nodes of the tree except 70 for the node separating strain M115 from the outbreak cluster and 40 and 41 for 2 nodes within the Papworth cluster 1 (*6*). SNPs, single-nucleotide polymorphisms.

(Figure 1). However, no information was available about any epidemiologic link between cystic fibrosis strain 47J26 to reported or unpublished outbreaks, and no clinical information was available about the patient from whom strain M18 was isolated. Therefore, both strains were excluded from the SNP analysis. Nevertheless, SNPs for these 3 strains at positions relevant to the outbreak strains are shown in the Technical Appendix (wwwnc.cdc.gov/EID/article/20/3/13-1106-Techapp1.xlsx).

A total of 293 identical SNPs in the core segments of Mugsy alignments were shared by the 10 outbreak strains but were different in available *M. abscessus* subsp. *massiliense* strains not related to outbreaks (Figure 2; online Technical Appendix). Of the 293 SNPs, 95 gave rise to nonsynonymous mutations in several genes, including virulence factors (mammalian cell entry and yrbE proteins), transcriptional regulators (TetR family), and lipid metabolism genes (online Technical Appendix). Eleven SNPs were shared only by Papworth and Seattle cystic fibrosis outbreak strains (n = 9), including nonsynonymous mutations in the preprotein translocase *secA1* and a putative lyase (Figure 2; online Technical Appendix). Sixteen SNPs were shared only by the 3 Seattle cystic fibrosis outbreak strains, including nonsynonymous mutations in a mycobacterial large membrane protein (MmpL) family involved in lipid transport and virulence (*34*) and genes involved in amino acid and energy metabolism (Figure 2; online Technical Appendix). Eighty-six SNPs were present only in strain CRM-0020 (soft tissue outbreak) from Brazil, including nonsynonymous mutations in an MmpL family protein; transcriptional regulators; and lipid, amino acid, and energy metabolism genes (Figure 2; online Technical Appendix).

Having shown high-level relatedness among Papworth and Seattle cystic fibrosis outbreak strains and their relatedness to the soft tissue outbreak strains from Brazil, we also searched for genomic regions ≥200 nt outside the core genome that were specific to subsets of isolates. A single region of ≈11.5 kb was unique to the Papworth cystic fibrosis isolates (n = 6) and encompassed 2 conserved hypothetical proteins, 2 phage integrase family proteins, and an MmpL family protein. Alignment of the MmpL family protein with distinct MmpL proteins described above for the Seattle cystic fibrosis outbreak and the Brazil soft tissue outbreak showed diversity at several amino acid residues in all 3 proteins.

No region was unique to the Seattle cystic fibrosis isolates (n = 3). The soft tissue isolate CRM-0020 from Brazil harbored several large unique regions, including a previously described broad–host-range IncP-1β plasmid (*35*) and 3 regions (contigs) of 5 kb, 10.6 kb, and 79 kb of unknown origin encoding almost exclusively hypothetical proteins.

We also searched for polymorphisms associated with macrolide and aminoglycoside resistance. The Papworth cystic fibrosis and Seattle cytic fibrosis outbreak set of strains showed an A2058C/G mutation in 23S rRNA, which conferred macrolide resistance (*36*) (A2058G in strains 2u and 28c representative of Papworth cluster 2 and the Seattle strains). Strains 19f, 14h, 12c, and 28a, representative of Papworth cluster 1, and Seattle strains shared the A1408G mutations in 16S rRNA, which conferred aminoglycoside resistance (*37*). None of these mutations were found in the soft tissue outbreak strains CRM-0020 and GO 06 from Brazil or the M18 strain.

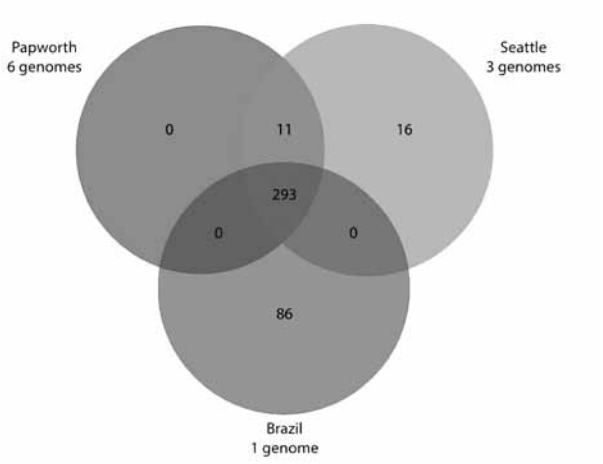


Figure 2. Venn diagram of core single-nucleotide polymorphisms (SNPs) shared by outbreak localities. Core segments of the Mugsy (*22*) alignment of the 20 *Mycobacterium abscessus* subsp. *massiliense* genomes (Table 1) were parsed for SNPs shared by different subsets of outbreak localities. Each field in the Venn diagram represents nucleotides that are identical among isolates of that field but different in other isolates represented on the diagram and non–outbreak-related *M. abscessus* subsp. *massiliense* strains 1S-151–0930, 5S-0817, M115, M139, M154, and the type strain CCUG 48898$^T$. Details on SNPs and genes they affect are shown in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/3/13-1106-Techapp1.xlsx).

### Diagnostic Tools for Identification of Outbreak Strains

In light of the possibility of a common ancestor and/or intercontinental transmission of strains, we identified SNPs in genes commonly used for identification of mycobacteria and an MLST scheme that could be used by clinical laboratories to assess relatedness of newly isolated strains to this global cluster. In the first approach, we retrieved *rpoB* sequences from the 6 genomes of representative strains of the Papworth cystic fibrosis outbreak and performed partial sequencing of the *rpoB* gene for selected isolates from the Seattle cystic fibrosis outbreak. We then compared these sequences with those of isolates from the outbreak in Brazil and unrelated clinical isolates comprising *M. abscessus* subsp. *abscessus*, *massiliense*, and *bolletii*, as well as other rapidly growing mycobacteria.

By using the *rpoB* gene MAB_3869c from the *M. abscessus* subsp. *abscessus* type strain as a reference (Table 2) described in the BRA-00 outbreak isolates from Brazil (*32,33*), we showed that Seattle (n = 4) and Papworth (n = 6) cystic fibrosis isolates carried the 2 *rpoB* SNPs (C→T at position 2569 and T→C at position 2760. However, none of the *M. abscessus* subsp. *abscessus* or subsp. *bolletii* or other rapidly growing mycobacterial isolates outside the *M. abscessus* group harbored this 2-SNP *rpoB* signature (Table 2). The second SNP (T→C substitution at position 2760) was present in several strains, but the combination of both *rpoB* SNPs (C→T at position 2569 and T→C at position 2760) was not present. Most of the 26 *M. abscessus* subsp. *massiliense* strains not related to outbreaks tested did not harbor this 2-SNP *rpoB* signature. However, 4 strains harbored this signature (Table 2) (*29,38*).

Multiple alignment of *rpoB* sequences among available *M. abscessus* subsp. *massiliense* genomes showed the absence of the 2-SNP *rpoB* signature in most strains. However, both SNPs were present in 1 strain not related to an outbreak (1S-151–0930) (Table 2).

Multiple alignment of *secA1* sequences among available *M. abscessus* subsp. *massiliense* genomes showed a G→T substitution at position 820 (by using the *secA1* gene MAB_3580c from the *M. abscessus* subsp. *abscessus* type strain) shared by the Papworth and Seattle cystic fibrosis outbreak strains but not by the soft tissue outbreak strains from Brazil or additional unrelated strains. Further analysis of *secA1* sequences from 12 *M. abscessus* subsp. *massiliense* identified by multitarget sequencing and PCR-based typing (*29,38*) showed a G→T substitution at position 820 in 2 strains unrelated to the outbreak (Table 2). Those 2 strains were included among the 4 strains that had the 2-SNP *rpoB* signature. Although the SNPs described for *rpoB* and *secA1* were not 100% specific markers for the outbreak strains, these SNPs could be used for first-level identification of newly isolated strains as possibly being related to cystic fibrosis clusters or soft tissue outbreak strains from Brazil to be confirmed by a second assay.

We also developed a simple MLST protocol that could be used as a second confirmatory assay. Alleles for each of 13 housekeeping genes (*cya*, *gdhA*, *argH*, *glpK*, *gnd*, *murC*, *pgm*, *pknA*, *pta*, *pur*, *rpoB*, *hsp65*, and *secA1*) were extracted and concatenated for each *M. abscessus* subsp. *massiliense* genome (Table 1), and the panel of concatenated sequences was used for construction of a neighbor-joining phylogenetic tree by using MEGA software. The Seattle and Papworth cystic fibrosis outbreak strains grouped together in the tree with cystic fibrosis strain 47J26 and isolate M18 from Malaysia (Figure 3). Thus, partial sequencing of *rpoB* and *secA1* gens, followed by 13-target MLST analysis, could be used to rule out isolates as belonging to these 2 cystic fibrosis clusters.

Table 2. Detection of *rpoB* and *secA1* SNP signature in the *Mycobacterium abscessus* group and rapidly growing mycobacteria*

| Strains | No. strains with SNP/no. tested (%) | | | |
|---|---|---|---|---|
| | *rpoB* T 2569 | *rpoB* C 2760 | *rpoB* T 2569 and *rpoB* C 2760 | *secA1* T 820 |
| MAB (CSU) | 0/44 (0) | 44/44 (100) | 0/44 (0) | NT |
| MAB (NIH) | 0/29 (0) | 29/29 (100) | 0/29 (0) | 0/29 (0) |
| MMA non-outbreak strain (CSU) | 1/14 (7) | 1/14 (7) | 0/14 (0) | NT |
| MMA non- outbreak strain (NIH) | 4/12 (33) | 10/ 12 (83) | 4/12 (33) | 2/12 (17) |
| MMA Seattle | 4/ 4 (100) | 4/4 (100) | 4/4 (100) | 3/3 (100) |
| MMA Brazil | 9/ 9 (100) | 9/9 (100) | 9/9 (100) | NT |
| MBO (CSU) | 0/11 (0) | 11/11 (100) | 0/11 (0) | NT |
| MBO (NIH) | 0/2 (0) | 2/2 (100) | 0/2 (0) | 0/2 (0) |
| Other RGM (CSU) | 0/42 (0) | 0/42 (0) | 0/42 (0) | NT |
| MMA type strain | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| MMA in silico data† | | | | |
| MMA UK | 6/6 (100) | 6/6 (100) | 6/6 (100) | 6/6 (100) |
| MMA Seattle | 3/3 (100) | 3/3 (100) | 3/3 (100) | 3/3 (100) |
| MMA Brazil | 2/2 (100) | 2/2 (100) | 2/2 (100) | 0/2 (0) |
| 47J26 | 1/1 (100) | 1/ 1 (100) | 1/1 (100) | 1/1 (100) |
| M18 | 1/1 (100) | 1/1 (100) | 1/1 (100) | 1/1 (100) |
| 1S-151–0930 | 1/1 (100) | 1/1 (100) | 1/1 (100) | 0/1 (0) |
| 5S-0817 | 0/1 (0) | 1/1 (100) | 0/1 (0) | 0/1 (0) |
| M115 | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| M139 | 0/1 (0) | 1/1 (100) | 0/1 (0) | 0/1 (0) |
| M148 | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| M154 | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| M156 | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| M159 | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| M172 | 0/1 (0) | 0/1 (0) | 0/1 (0) | 0/1 (0) |
| Asan 50594 | 0/1 (0) | 1/1 (100) | 0/1 (0) | 0/1 (0) |

*SNP, single-nucleotide polymorphism; MAB, *M. abscessus* subsp. *abscessus*; NT, not tested. CSU, Colorado State University; NIH, National Institutes of Health; MMA, *M. abscessus* subsp. *massiliense*; MBO, *M. abscessus* subsp. *bolletii*; RGM, rapidly growing mycobacteria.
†Information collected from available whole genome sequencing data.

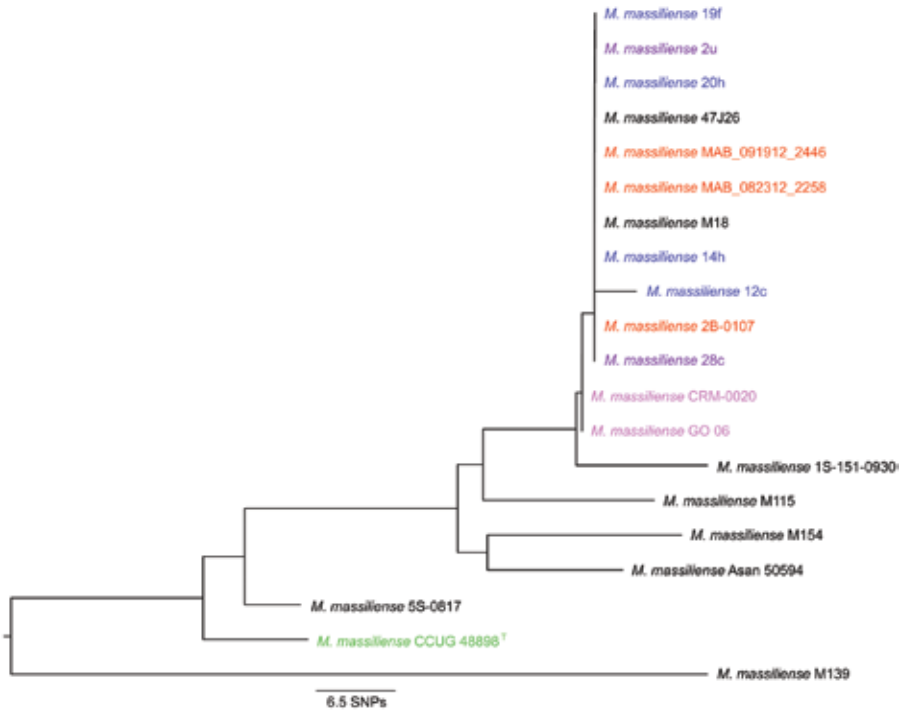


Figure 3. Neighbor-joining phylogenetic tree based on 13-target multilocus sequences types from 20 *Mycobacterium abscessus* subsp. *massiliense* genomes. Electronic PCR was performed on the *M. abscessus* subsp. *massiliense* genomes listed in Table 1 by using primer pairs for 13 housekeeping genes (*cya*, *gdhA*, *argH*, *glpK*, *gnd*, *murC*, *pgm*, *pknA*, *pta*, *pur*, *rpoB*, *hsp65*, and *secA1*), including new primers designed as part of this study. Nucleotide sequences from each gene were concatenated for each genome and aligned by using ClustalW (*31*), and the core alignment was used for construction of a midpoint-rooted neighbor-joining phylogenetic tree by using MEGA (*24*). Strains from an outbreak of *M. abscessus* subsp. *massiliense* infections at a cystic fibrosis center in Seattle, Washington, USA, are indicated in red; strains from an outbreak of *M. abscessus* subsp. *massiliense* infections at a cystic fibrosis center in Papworth, UK, are indicated in blue (cluster 1) and purple (cluster 2); strains from Brazil are indicated in magenta; and the *M. abscessus* subsp. *massiliense* type strain is indicated in green. The longer branch length for Papworth isolate 12c was caused by low-quality nucleotides (single-nucleotide polymorphisms [SNPs]) located at the edge of Velvet contigs.

## Discussion

The implications of this study are extensive. Currently, most experts recommend identifying isolates of *M. abscessus* to subspecies level (*39*). This report further corroborates these recommendations and places even greater pressure on clinical laboratories to fully identify *M. abscessus* subspecies *massiliense*.

Strains from the 2 cystic fibrosis outbreaks showed high-level relatedness (4,264,844 nt core genome alignment size, 11 shared unique SNPs) with each other and major-level relatedness (4,231,390 nt core genome alignment size) with soft tissue epidemic strains from Brazil. Genomic features shared between strains from all 3 outbreaks might make them more transmissible, whether from patient to patient (directly or indirectly as in cystic fibrosis outbreaks) or from a common source, as in soft tissue infections. However, the soft tissue strain from Brazil had the largest number of unique SNPs (86) not shared with either of the cystic fibrosis outbreak strains, harbored an IncP-1β plasmid, and did not show mutational resistance to amikacin or clarithromycin. We speculate that some of these specific genomic traits may be favorable for the successful establishment of epidemic soft tissue infections.

A previous study did not detect a common source or person-to-person transmission of the *M. abscessus* group among cystic fibrosis patients and suggested that it may not be necessary to segregate persons infected or colonized with *M. abscessus* from those who are not infected or colonized (*40*). Our findings emphasize the necessity of screening all isolates of *M. abscessus* subsp. *massiliense* recovered from patients with cystic fibrosis for relatedness to outbreak strains in an effort to prevent future outbreaks. Because of evidence supporting patient-to-patient transmission of multiple different respiratory tract organisms, the Infection Control Guidelines (currently in draft form for public comment) of the United States Cystic Fibrosis Foundation (CFF) (www.cff.org/LivingWithCF/Webcasts/ArchivedWebcasts/Germs/#Infection_Prevention_and_Control_Policy_Update) have been recently changed. Patients with cystic fibrosis are advised not to attend indoor meetings with other cystic fibrosis patients (CFF and Infection Prevention and Control Guidelines 2013). In addition, screening of all cystic fibrosis patients in the United States

at least annually for mycobacteria is now recommended (CFF and Infection Prevention and Control Guidelines 2013) to enable early treatment if the organism is detected.

It remains unclear why intercontinental organisms are so closely related. One hypothesis is that direct patient contact led to transmission. The Seattle index case-patient traveled to British Columbia, Canada, before and after acquiring mycobacterial infection, to Oregon before mycobacterial infection, and to Atlanta, Georgia, and Bethesda, Maryland, after mycobacterial infection. However, the patient did not report any contact with other cystic fibrosis patients at these destinations. A second hypothesis is that the mycobacterial strain could have been carried by persons with cystic fibrosis who were clinically well. A third hypothesis is that there was an independent selection of *M. abscessus* subsp. *massiliense* clones in the cystic fibrosis airway milieu on both sides of the Atlantic Ocean toward potentially more transmissible lineages. Availability of additional whole-genome sequencing data tracking the global epidemiology of the *M. abscessus* group may help differentiate between these scenarios. In addition, this data will help delineate global clusters of *M. abscessus* subsp. *massiliense* strains with potentially higher transmissibility.

## Addendum

Recent whole-genome data show deep genetic separation of 3 subspecies, ruling against grouping *M. massiliense* and *M. bolletii* under *M. abscessus* subsp. *bolletii*.

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# Hendra Virus Vaccine, a One Health Approach to Protecting Horse, Human, and Environmental Health

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In recent years, the emergence of several highly pathogenic zoonotic diseases in humans has led to a renewed emphasis on the interconnectedness of human, animal, and environmental health, otherwise known as One Health. For example, Hendra virus (HeV), a zoonotic paramyxovirus, was discovered in 1994, and since then, infections have occurred in 7 humans, each of whom had a strong epidemiologic link to similarly affected horses. As a consequence of these outbreaks, eradication of bat populations was discussed, despite their crucial environmental roles in pollination and reduction of the insect population. We describe the development and evaluation of a vaccine for horses with the potential for breaking the chain of HeV transmission from bats to horses to humans, thereby protecting horse, human, and environmental health. The HeV vaccine for horses is a key example of a One Health approach to the control of human disease.

Hendra virus (HeV) is an emerging zoonotic paramyxovirus for which natural reservoirs are the 4 species of flying fox (*Pteropus* bats) found on mainland Australia (*1*). HeV was discovered in 1994, and since then, infections have occurred in 7 humans, 4 of whom died. Each case-patient had a strong epidemiologic connection to similarly affected horses through exposure to equine secretions late in the incubation period, during terminal illness, or at the time of postmortem examination of infected animals (*2*): no human case of HeV infection has been attributable to direct spillover from bats (*3*).

HeV infection in the bat host appears to be asymptomatic (*1*); however, in humans and horses there is evidence of initial virus replication in the nasopharynx that progresses through a viremic phase during which the virus spreads to major organ systems, resulting in disseminated endothelial cell infection, vasculitis, encephalitis, and pneumonia (*4*–*7*). There is no licensed anti-HeV therapeutic drug for use in any species. Experimental exposure of horses to HeV/Australia/Horse/2008/Redlands under Biosafety Level 4 (BSL-4) conditions identified comparatively low gene copy numbers in nasal secretions early in the incubation period. However, gene copy numbers increased exponentially with the onset of fever, when viral genome could also be recovered from blood, oral secretions, urine, and feces (*6*). Rapid progression of clinical signs, as observed in equine field cases of this disease, led to euthanasia of experimental animals on humane grounds. Viral RNA was recovered from all tissues sampled at postmortem examination, and virus was reisolated from lung, brain, lymphoid tissues, and kidney (*6*). In accordance with epidemiologic observations (*2*), it was concluded that HeV-infected horses in the immediate presymptomatic or symptomatic stages of disease pose a high risk for transmission of HeV to humans. This risk is then exacerbated because it is symptomatic horses that come to the attention of veterinarians, leading to various

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clinical investigations (e.g., respiratory tract endoscopy) that may facilitate human exposure to virus.

During 1994–2010, there were a total of 14 HeV outbreaks, including those with the 7 human infections. Then, in 2011, for reasons that are as yet poorly understood, an unprecedented 18 equine incidents, some involving >1 horse, occurred within a 3-month period and over an expanded geographic range, emphasizing that HeV was an unmanaged emerging disease (*3*). These events were accompanied by a marked rise in the number of HeV-related media reports. The reports had an increasingly politicized focus on the role (and control) of flying foxes as carriers of HeV (*8*) and a deemphasis of the critical role played by horses in HeV transmission to humans.

Heightened public awareness of the risk that infected horses posed to humans persisted and was paralleled by increased numbers of veterinarians leaving equine practice because of personal safety and liability concerns (*9*). The considerable investment in education and improved infection control measures that had been implemented did not effectively mitigate perceptions around the risks associated with the routine veterinary care of horses (*10*).

The actual mechanism of HeV transmission from bats to horses is probably complex and dependent upon socioeconomic, environmental, and ecologic factors (*11*), and there is currently no straightforward solution for preventing transmission. Eradication of flying foxes would pose extraordinary operational challenges, notwithstanding attendant moral, ethical, and environmental issues, and eliminating the interface between bats and horses is impractical for periurban and rural communities.

The most direct approach for reducing the risk posed to humans by HeV-infected horses would be implementation of a strategy that will lead to suppression of virus replication in horses. We describe the development and evaluation of a vaccine for horses with the potential for breaking the chain of HeV transmission from bats to horses to humans, thereby protecting horse and human health. The emergence of several highly pathogenic zoonotic diseases in humans in recent years has led to a renewed emphasis on the interconnectedness of human, animal, and environmental health, otherwise known as One Health. The HeV vaccine for horses, Equivac HeV (Zoetis, Parkville, VIC, Australia), is a key example of a One Health approach to the control of human disease (*12*).

## Materials and Methods

### Animals, Accommodation, Handling, and Biosafety

For efficacy studies, up to 3 female horses at a time were housed in single pens under BSL-4 conditions meeting the Victorian Bureau of Animal Welfare Code of Practice for the Welfare of Horses (www.dpi.vic.gov.au/agriculture/about-agriculture/legislation-regulation/animal-welfare-legislation/codes-of-practice-animal-welfare/code-welfare-of-horses). One of the sides of each pen was able to be moved in toward the horse on a ratchet mechanism, allowing staff close access to the horses, as required, over the side of the pen without the need for them to enter the pen itself (*13*). Room temperature was maintained at 22°C with 15 air changes/h; humidity ranged from 40% to 60%. Horses were fed a mixture of lucerne (alfalfa) and grass hay, concentrates, and specified fruit and vegetables. On the day before HeV exposure, an indwelling jugular catheter was sutured in position, and an intrauterine temperature data-logger was placed into each horse. All vaccinated horses were euthanized electively on day 7, 8, or 9 after challenge; unvaccinated horses were euthanized upon reaching a predetermined humane endpoint (6–9 days after vaccination). The humane end point was defined as fever for up to 48 h accompanied by increased respiratory rate, dyspnea, depression, ataxia, or pressing the head against the side of the stall. Euthanasia was conducted by intravenous injection of a barbiturate following sedation with intravenous detomidine and butorphanol.

Ferrets and guinea pigs used as controls in efficacy studies to confirm pathogenicity of the inoculum were housed in pairs in the BSL-4 facility, given species-appropriate dry rations and dietary treats, and provided with water ad libitum. For virus challenge and sampling, they were immobilized by intramuscular injection of a mixture of ketamine hydrochloride (3 mg/kg) and medetomidine (30 g/kg). The effects of medetomidine were reversed by intramuscular injection of atipemazole (15 g/kg). While in the BSL-4 animal room, staff wore fully encapsulated suits with an external air supply.

As appropriate, animal studies were endorsed by the CSIRO Australian Animal Health Laboratory Animal Ethics Committee and/or Commonwealth Serum Laboratories /Zoetis Animal Ethics Committee. Work using gamma-irradiated HeV soluble G (HeVsG) glycoprotein produced in Chinese hamster ovary (CHO) cells was done under Australian Quarantine and Inspection Service in vivo permit number 2012/012, and work using non-gamma–irradiated HeVsG glycoprotein produced in 293F human embryonic kidney was done under Australian Quarantine and Inspection Service in vivo permit number 2010/027. All clinical trials were conducted under the Australian Pesticides and Veterinary Medicines Authority research permits PER 7250, PER 13169, PER 13247, and PER 13418.

### Vaccine Preparation

A subunit vaccine containing recombinant HeVsG glycoprotein (*14*) was formulated in a proprietary adjuvant (Zoetis). For vaccine formulation, HeVsG glycoprotein was produced by using a Chinese hamster ovary

(CHO) or a 293F human embryonic kidney cell expression system (*15*) with 1 of 2 different HeVsG glycoprotein preparations: 1) affinity-purified sG glycoprotein (293F cells) or 2) clarified sG containing cell culture supernatant (CHO cells). Vaccines for initial efficacy studies in target species were formulated with 50 μg or 100 μg of affinity-purified sG glycoprotein. All subsequent vaccines were formulated with clarified CHO cell culture supernatant that was then gamma irradiated. The change of the expression system from 293F cells to CHO cells was driven by the need for higher antigen yields, and equivalence was supported by laboratory analysis of the expressed antigens from the 2 systems and a comparison study in ferrets. Vaccine formulations used in efficacy studies are summarized in Table 1.

### Immunization

All immunizations comprised two 1-mL doses administered intramuscularly 3 weeks apart, unless stated otherwise. In the efficacy studies, 7 horses (V1, V2, and V6–V10) received vaccine containing 100 μg of HeVsG glycoprotein/dose and 3 horses (V3–V5) received 50 μg of HeVsG glycoprotein/dose (Table 1).

### Animal Infection

Horses in the efficacy studies were exposed oronasally to $2 \times 10^6$ 50% tissue culture infectious doses of a low-passage HeV isolate (Hendra virus/Australia/Horse/2008/Redlands). Horses V1–V7 were challenged 28 days after the second vaccination, and horses V8–V10 were challenged 194 days after the second vaccination. Horses V8–V10 were selected from 29 vaccinated horses in a larger field efficacy and safety study on the basis of temperament and for having the lowest serum neutralization titers in the group at the time. Overall, 4 efficacy tests were completed; 2 vaccinated horses were used in the first test, 3 were used in the second, 2 were used in the third, and 3 were used in the fourth. For the 4 tests, a pathogenicity control for the inoculum was provided by 1 horse (test 1), 4 guinea pigs (test 2), 2 ferrets (test 3), and 2 ferrets (test 4). Guinea pigs and ferrets each received 50,000 50% tissue culture infectious doses of the same virus preparation that was used in the horses; guinea pigs received the dose by intraperitoneal injection, and ferrets received the dose by the oronasal route. Experience has shown that these doses and routes of administration were expected to be lethal in ≥25% of guinea pigs and 100% of ferrets. Exposure conditions for 3 additional unvaccinated control horses were equivalent to those used in both vaccinated horses and the inoculum-control horse and have been described (*6*).

### Sample Collection and Analysis

During efficacy studies, nasal, oral, and rectal swab samples; urine and feces samples; and blood samples (in EDTA) were collected from the horses before virus exposure and then daily until the animals were euthanized. Swab samples were collected in duplicate into 1 mL of phosphate-buffered saline for virus isolation or into 800 mL of MagMax Lysis/Binding Solution (Ambion, Austin, TX, USA) for RNA extraction. For urine and EDTA blood samples, 100 mL of fluid was added to 260 mL of the lysis/binding solution. At postmortem examination, the following tissues were collected for viral genome detection, virus isolation, histopathology, and immunohistochemistry according to (*15*): adrenal gland, bladder, brain (including olfactory pole), cerebrospinal fluid, guttural pouch, heart, kidney, large intestine, liver, lung, lymph nodes (bronchial, inguinal, intermandibular, mandibular, renal), meninges, nasal turbinates, ovaries, pharynx, small intestine, spinal cord, spleen, sympathetic nerve, trigeminal ganglion, and uterus. The following analyses were conducted as described (*15*): quantitative reverse transcription PCR for the detection of the HeV N gene, histology, immunohistology, serum neutralization test, and virus isolation.

Table 1. Details, by efficacy trial number, of subunit vaccine formulations containing recombinant Hendra virus soluble G glycoprotein*

| Trial no., horse identification | Hendra virus soluble G glycoprotein specification | | | Challenge, days after vaccination | Viral infectivity control | |
|---|---|---|---|---|---|---|
| | Source | Irradiation | Dose, μg | | Species | No. |
| 1 | | | | | Horse | 1 |
| V1 | 293F HEK | No | 100 | 21 | | |
| V2 | 293F HEK | No | 100 | 21 | | |
| 2 | | | | | Guinea pig | 4 |
| V3 | 293F HEK | No | 50 | 21 | | |
| V4 | 293F HEK | No | 50 | 21 | | |
| V5 | 293F HEK | No | 50 | 21 | | |
| 3 | | | | | Ferret | 2 |
| V6 | CHO | Yes | 100 | 21 | | |
| V7 | CHO | Yes | 100 | 21 | | |
| 4 | | | | | Ferret | 2 |
| V8 | CHO | Yes | 100 | 194 | | |
| V9 | CHO | Yes | 100 | 194 | | |
| V10 | CHO | Yes | 100 | 194 | | |

*HEK, human embryonic kidney cells; CHO, Chinese hamster ovary cells.

## Results

Vaccine efficacy in immunized horses was assessed against the clinical, virologic, and pathologic features of HeV infection in 4 unvaccinated control horses. Infection characteristics for 3 of these unvaccinated animals have been described (*6*); data from the fourth control animal was gathered as part of the current work. In that fourth control, onset of fever accompanied by a rising heart rate was noted on postchallenge day 6. On postchallenge day 7, the horse became clinically depressed, its temperature and heart rate continued to rise, and it was euthanized. Gross postmortem findings included pleural thickening and moderate dilation of the lymphatic vessels on the ventral 10 cm of the cardiac lung lobes. Histologic examination revealed systemic vasculitis affecting the lung (Figure 1, panel A), spleen, kidney, nasal epithelium, lymph nodes, and brain; alveolitis; and lymphadenitis. HeV antigen was identified in endothelial cells and vascular walls within lung, brain (Figure 1, panel B), nasal epithelium, lymph nodes, spleen, kidney, liver, myocardium, salivary gland, pharynx, small intestine, uterus, ovary, and adrenal gland, as well as in myocardial fibers and glomeruli.

Viral RNA from this fourth control horse was detected in nasal swabs collected on postchallenge day 3 (Table 2; summarized in Table 3) and also in blood collected immediately before the onset of fever. After onset of fever, but before development of other clinical signs of illness, HeV RNA was also detected in the oral swab sample. On the day of euthanasia, genome was detected in oral and nasal swab samples, blood, rectal swab, and urine samples; however, virus was not reisolated from any sample collected before postmortem examination. Viral RNA was detected in all tissues sampled at postmortem examination except cerebrospinal fluid. Reisolation of virus was attempted for all tissues: HeV was recovered from lung, submandibular lymph node, small intestine, large intestine, and adrenal gland.

In a series of vaccine efficacy studies, 10 horses were immunized with HeVsG glycoprotein and then exposed to an otherwise lethal dose of HeV by the oronasal route. Each study also included a pathogenicity control for the virus inoculum. In the first of these, the pathogenicity control was the fourth control horse described above. Together with historical data gathered from 3 horses following their exposure to HeV under equivalent experimental conditions (*5*), data from this horse completed the requirements of the Australian Pesticides and Veterinary Medicines Authority for defining the horse infection model. In subsequent studies, guinea pigs or ferrets were used as pathogenicity controls to maximize the number of vaccinated horses that could be accommodated in the BSL-4 facility. These animals duly displayed signs, lesions, tissue antigen and viral genome distribution, and virus reisolation data consistent with acute HeV infection.

In contrast to unvaccinated control horses, vaccinated horses remained clinically healthy during the observation period after exposure to HeV. Following elective euthanasia at the time of predicted peak viral replication, there was no gross or histologic evidence of HeV infection in vaccinated horses; all tissues examined were negative for viral antigen by immunohistochemistry; and viral genome was not recovered from any tissue, including nasal turbinates, pharynx, and guttural pouch (Table 3). For 9 of 10 vaccinated horses, viral RNA was not detected in daily nasal, oral, or rectal swab specimens or from blood, urine, or feces samples collected before euthanasia, and virus was not reisolated from any of these clinical samples. For 1 (V9) of 3 horses exposed to HeV 6 months after completing the vaccination course, low viral gene copy numbers were detected in nasal swab samples collected on postchallenge days 2–4 and 7 (Figure 2); this finding was consistent with self-limiting local replication. Virus was not reisolated from these samples.

Serum neutralization titers before HeV challenge ranged from 128/256 to >4,096 for horses V1–V7 when challenged 21 days after the second vaccination and from 16 to 32 for horses V8–V10 when challenged 6 months after the second vaccination (Table 3). At the time of euthanasia, no rise in antibody titer was detected in any vaccinated horse following exposure to HeV.

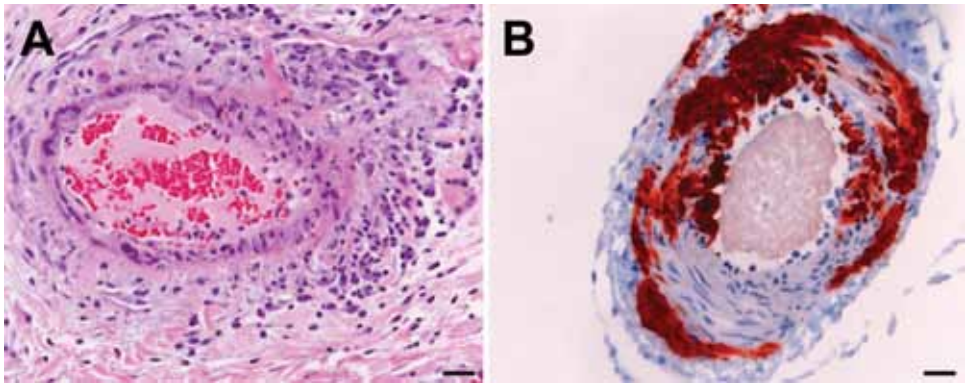


Figure 1. Histologic and immunohistologic findings in Hendra virus–infected horse tissue. A) Hematoxylin and eosin staining shows systemic vasculitis affecting the lung. B) Immunohistologic examination, using polyclonal rabbit anti-Nipah N protein, indicates Hendra virus antigen in a blood vessel in the brain. Scale bars represent 50 μm.

Table 2. Quantitative reverse transcription PCR detection of Hendra virus N gene in samples collected daily from control horses*

| Horse no., sample | $log_{10}$ relative copy number of Hendra virus N RNA, dpc | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| 1† | | | | | | | | | | |
| Blood | – | – | – | – | – | –0.2 | 1.4 | 1.7 | | |
| Urine | – | – | – | – | – | – | –0.8 | 1.1 | | |
| Feces | – | – | – | – | – | – | – | –0.7 | | |
| Nasal swab | – | – | –0.3 | –0.2 | 1.4 | 1.6 | 1.4 | 1.7 | | |
| Oral swab | – | – | – | – | – | –0.1 | 1.3 | 1.3 | | |
| 2‡ | | | | | | | | | | |
| Blood | – | – | – | – | 0.5 | 2.6 | 3.0 | | | |
| Urine | – | – | – | – | – | 0.3 | 1.7 | | | |
| Feces | – | – | – | – | – | –0.05 | 2.0 | | | |
| Nasal swab | – | – | 1.2 | 2.0 | 1.6 | 3.5 | 2.5 | | | |
| Oral swab | – | – | – | – | – | 0.4 | 1.5 | | | |
| 3‡ | | | | | | | | | | |
| Blood | – | – | – | – | – | – | 1.5 | 2.8 | 2.9 | 3.4 |
| Urine | – | – | – | – | – | – | – | 1.8 | 1.7 | 3.0 |
| Feces | – | – | – | – | – | – | – | 1.5 | 1.7 | 2.1 |
| Nasal swab | – | – | 1.7 | 2.5 | 1.2 | 2.4 | 3.0 | 3.8 | 3.7 | 2.0 |
| Oral swab | – | – | – | – | – | – | – | 1.9 | 1.9 | 2.3 |
| 4‡ | | | | | | | | | | |
| Blood | – | – | – | – | – | 0.1 | 1.9 | 2.5 | 3.0 | |
| Urine | – | – | – | – | – | – | 0.07 | 0.5 | 2.1 | |
| Feces | – | – | – | – | – | – | 1.3 | 2.4 | 2.1 | |
| Nasal swab | – | – | 0.3 | – | – | – | – | 1.6 | 2.5 | |
| Oral swab | – | – | – | – | – | – | 0.2 | 1.2 | 1.6 | |

*Duplicate samples were obtained and tested by reverse transcription PCR. Cycle threshold values were converted to relative copy numbers by using a standard curve of a sample with a known copy number. dpc, days after challenge. – indicates a negative result; blank space indicates no sample was tested.
†N gene data for horse 1 was obtained from the current study.
‡N gene data for horses 2–4 are unpublished data from a previous study (*6*).

## Discussion

The formal launch of the HeV horse vaccine in November 2012 represents the culmination of multiple studies conducted in several animal infection models over the course of many years. Studies using Nipah virus in cats (*16*,*17*) and monkeys (*18*) and HeV in ferrets (*15*) provided strong evidence that a HeVsG glycoprotein subunit–based vaccine could prevent not only disease but often infection in animals exposed to otherwise lethal doses of Nipah virus or HeV. Where evidence of low-level virus replication did occur in secretions, it was transient and unaccompanied by the development of clinical illness, and virus was not isolated from the secretions.

The henipavirus surface-expressed G glycoprotein has the critical role of initiating infection by binding to receptors on host cells, and antibodies directed against this protein can neutralize virus (*19*). Earlier reports have shown that passive immunotherapy with antibody to the G or F glycoprotein of HeV or Nipah virus alone can prevent fulminating disease (*20*): G glycoprotein–specific human monoclonal antibody prevented Nipah virus disease in ferrets (*21*) and HeV infection in African green monkeys (*22*); and F or G glycoprotein–specific monoclonal or polyclonal antibodies prevented HeV and Nipah virus disease in hamsters (*23–25*). Thus it is likely that, as seen for other paramyxoviruses with a viremic infection phase (e.g., measles and mumps), antibodies to the G and F glycoproteins play a major role in protection provided by HeVsG glycoprotein vaccination (*26–28*).

In the studies reported here, we show that 2 doses of a commercially formulated HeVsG glycoprotein subunit–based vaccine prevented infection in 7 of 7 horses exposed to HeV at least 21 days after the second vaccine dose; this finding is in contrast to that for unvaccinated control horses. Similar results were obtained for 2 of 3 horses exposed to HeV 6 months after vaccination. In the third horse, which also remained clinically healthy, evidence of HeV replication was limited to low-level transient detection of viral genome (but not virus) from the nasal cavity. In assessing the field significance of this observation, the following must be noted: the experimental horses were exposed to considerably higher levels of HeV than have been recovered from flying foxes (*1*), higher levels of viral genome were routinely found in the nasal secretions of nonimmunized horses, and all human infections have been acquired from animals in which clinical disease developed. It is reasonable to suggest that the higher transmission risk that is clearly associated with such horses is a consequence of not only increased viral load but also of the illness itself: it is the clinically ill horse that promotes increased human–animal contact through diagnostic investigations and administration of nursing care. We conclude that the level

Table 3. Summary of sample analysis data from 10 horses in the 4 efficacy trials*

| Trial no, horse ID | Prechallenge antibody titer | Euthanized, dpc | Genome detection, sample, no. dpc | | | | | | | Viral infectivity control | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | PM tissue | Oral swab | Rectal swab | Nasal swab | Urine | Feces | Blood | Specimen | No. died/no. total |
| 1 | | | | | | | | | | Horse | 1/1 |
| V1 | 512, 1,024 | 8 | – | – | – | – | – | – | – | | |
| V2 | 512, 1,024 | 9 | – | – | – | – | – | – | – | | |
| 2 | | | | | | | | | | Guinea pig | 1/4 |
| V3 | 2,048, 4,096 | 7 | – | – | – | – | – | – | – | | |
| V4 | 128, 256 | 8 | – | – | – | – | – | – | – | | |
| V5 | >4,096, >4,096 | 9 | – | – | – | – | – | – | – | | |
| 3 | | | | | | | | | | Ferret | 2/2 |
| V6 | >4,096, >4,096 | 7 | – | – | – | – | – | – | – | | |
| V7 | >4,096, >4,096 | 8 | – | – | – | – | – | – | – | | |
| 4 | | | | | | | | | | Ferret | 2/2 |
| V8 | 32, 32 | 7 | – | – | – | – | – | – | – | | |
| V9 | 16, 32 | 8 | – | – | – | 2–4, 7 | – | – | – | | |
| V10 | 16, 32 | 9 | – | – | – | – | – | – | – | | |

*A 100-μg dose of Hendra virus soluble G glycoprotein was used in trials 1, 3, and 4; a 50-μg dose was used in trial 2. Genome was detected by PCR. ID, identification; dpc, days after challenge; PM, postmortem. – indicates a negative result.

and pattern of virus replication in the 1 vaccinated horse do not meet the epidemiologic criteria presently associated with transmission of infection to humans.

In previous henipavirus vaccine efficacy studies in cats and ferrets, a neutralizing antibody titer of 32 was shown to be protective against the development of clinical disease (*17*). In the horse efficacy studies, the 3 horses with prechallenge antibody titers of 16 or 32 were similarly protected from clinical illness. However, we caution that any correlation between antibody titer at the time of exposure to virus and levels of subsequent protection against infection and disease is unlikely to be linear; it is possible that animals with even lower titers will have epidemiologically meaningful protection against HeV exposure occurring in the field, not least because of stimulation of immunological memory. Additional studies assessing the duration of protection are planned, and the outcome of these will further inform recommendations regarding booster vaccination.

As expected, initial uptake of the HeVsG glycoprotein subunit–based vaccine was strongest in the area with the highest perceived risk for HeV infection, namely coastal Queensland, Australia. In other regions where HeV infection of horses has not been reported, there is understandably more uncertainty regarding the value of vaccination as part of horse preventative health programs. Any reluctance to vaccinate horses against HeV that is based on assessment of risk is probably exacerbated by several factors, including the novelty of the vaccine roll-out process to the Australian horse industry, a (mistaken) perception that fast-tracking vaccine release involved overlooking key safety and efficacy issues, the lack of published data on safety in pregnant mares, reluctance of certain industry sectors to vaccinate because of import restrictions on HeV-seropositive horses, and cost. Although it is likely that each of these barriers will diminish over time, our experiences may assist the development of road maps to guide the future release of vaccines against BSL-4 pathogens that are associated with highly sporadic disease events and where the decision to vaccinate is in the hands of the persons whom vaccination was designed to protect.

Several recently emerged zoonotic viruses, including HeV, Nipah, Ebola, and Marburg viruses, are classified as BSL-4 agents because of their ability to cause severe illness or death in humans and because there have been no effective vaccines or postexposure treatments to protect against the diseases they cause. The vaccine against HeV (Equivac HeV) is a commercially deployed vaccine developed against a BSL-4 agent and is the only licensed treatment for henipavirus infection.

Development of vaccines against BSL-4 agents for use in humans requires that the US Food and Drug Administration implement the animal rule, which requires that such vaccines first be tested for efficacy in at least 2 animal models (*29*). As a veterinary vaccine, Equivac

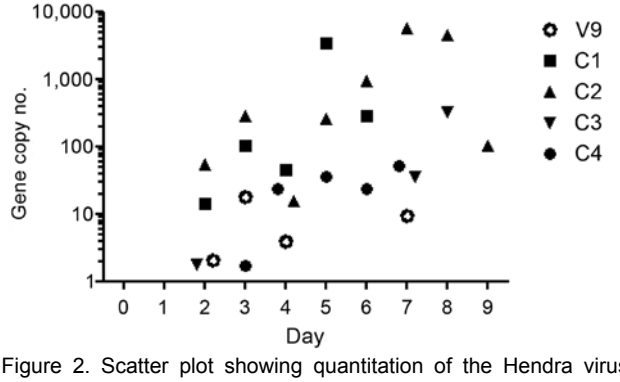


Figure 2. Scatter plot showing quantitation of the Hendra virus N gene in nasal swab samples from 1 vaccinated horse (V9) and 4 control horses (C1–C4); controls were challenged but not vaccinated. Days represent days after challenge.

HeV did not need to meet this requirement, and it was both cheaper and faster to produce than a vaccine intended for human use. At the same time, the vaccine is expected to provide a substantial health benefit to humans. In so doing, this vaccine encapsulates the spirit of a One Health approach, not just in terms of the interconnectedness of human and animal health but also with respect to environmental health. One consequence of the recent HeV outbreaks was a move to eradicate bat populations, despite their crucial environmental roles in pollination and reduction of the insect population. Successful deployment of the HeV vaccine, with a targeted reduction in the risk for acute disease events in horses and humans, should help reduce the current momentum toward the setting of control policies with potential adverse effects on the environment. Furthermore, the increasing evidence for henipaviruses and henipa-like viruses in bats in other areas (*30–32*) raises the possibility of future henipavirus outbreaks. The current HeVsG glycoprotein vaccine technology provides a platform for the rapid development of related vaccines to counter future emergent threats.

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C.C.B. is a US federal employee and an inventor (on pending US patents and Australian patent 2005327194, pertaining to soluble forms of Hendra and Nipah G glycoproteins); patent assignees are the United States government, as represented by the Department of Health and Human Services, and the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc.

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# etymologia

## *Mycobacterium abscessus* subsp. *bolletii*

From the Latin *ab-* ("away") + *cedere* ("to go"), an abscess is named for the notion that humors leave the body through pus. *Mycobacterium abscessus* was first isolated from gluteal abscesses in a 62-year-old patient who had injured her knee as a child and had a disseminated infection 48 years later. The species *M. bolletii*, named after the late microbiologist and taxonomist Claude Bollet, was described in 2006. In current taxonomy, *M. bolletii* and *M. massiliense* (named for Massilia, the ancient Greek and Roman name for Marseille, where the organism was isolated) have been incorporated into *M. abscessus* subsp. *bolletii*.

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# Possible Role of Songbirds and Parakeets in Transmission of Influenza A(H7N9) Virus to Humans

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Avian-origin influenza A(H7N9) recently emerged in China, causing severe human disease. Several subtype H7N9 isolates contain influenza genes previously identified in viruses from finch-like birds. Because wild and domestic songbirds interact with humans and poultry, we investigated the susceptibility and transmissibility of subtype H7N9 in these species. Finches, sparrows, and parakeets supported replication of a human subtype H7N9 isolate, shed high titers through the oropharyngeal route, and showed few disease signs. Virus was shed into water troughs, and several contact animals seroconverted, although they shed little virus. Our study demonstrates that a human isolate can replicate in and be shed by such songbirds and parakeets into their environment. This finding has implications for these birds' potential as intermediate hosts with the ability to facilitate transmission and dissemination of A(H7N9) virus.

The emergence of novel influenza strains from the avian reservoir remains a constant threat to human and animal health, as was recently illustrated by human infections with novel and wholly avian influenza A(H7N9) viruses in China. These viruses show little virulence in birds but can cause severe illness in humans (*1*,*2*). Of the 134 confirmed human cases reported as of August 2013, >30% were fatal (*3*,*4*). In the 3 index case-patients, the illness progressed to acute respiratory distress syndrome and death (*1*), and most persons with confirmed infections required hospital care (*2*,*5*). Retrospective epidemiologic analyses showed >75% of affected patients had had contact with domestic poultry (*6*,*7*), a common source of zoonotic transmission of influenza (*8*). Several of the A(H7N9) virus internal genes (polymerase basic protein [PB] 1, matrix, nonstructural protein, and nucleoprotein) originated from the H9N2 subtype commonly found in chickens. When chickens and quail were inoculated with A(H7N9) isolated from humans, they shed the viruses to high titers but had little or no clinical disease (*9*,*10*). Thus, poultry appears to be a reservoir for A(H7N9) viruses and a source of human infections. Yet, multiple lines of evidence suggest avian species other than the usual suspects (waterfowl and poultry) contributed to the emergence of these novel H7N9 viruses: first, H7N9 has been isolated from nonpoultry birds (pigeons) in Chinese live-bird markets (*11*); second, 2 genes (PA, PB2) in an initially characterized human isolate (A/Anhui/1/2013) were most closely related to viruses isolated from bramblings (finch-like birds of the large order Passeriformes) (*12*); and third, the matrix, polymerase acidic protein [PA], PB1 and PB2 gene segments from additional human isolates appear to have been donated by A/brambling/Beijing/16/2012 (H9N2)-like virus(es) (*13*). Therefore, songbirds and other small, terrestrial birds could have been directly involved in the genesis of novel A(H7N9) viruses and subsequent infection in humans.

Songbirds are common household pets and are in close contact with humans and domesticated animals. Their wild counterparts also are likely to interact with poultry in backyard farms and in many farming sectors (*14*,*15*). Consequently, we examined the replication and transmission of the human isolate A/Anhui/1/2013 (H7N9) in wild and

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domesticated small birds. A/Anhui/1/2013 was isolated from one of the initially reported human case-patients (*1*) and is closely related to many of the avian isolates that have been recovered (*12*). For this study, we chose 3 species of Passeriformes (zebra finches, society finches, and sparrows), which are related to the bramblings described previously. We also studied the parakeet (budgerigar; order Psittaciformes), a bird found in the wild and in households as a pet, that is known to support the replication of other subtypes of influenza (*16*–*18*). The study was conducted during June and July 2013 at St. Jude Children's Research Hospital (Memphis, TN, USA).

## Methods

### Virus and Facilities

A/Anhui/1/2013 (H7N9), A/Vietnam/1203/04 (H5N1), and A/songbird/Hong Kong/SB102/2001 (H3N8) viruses were propagated and titrated in chicken eggs as described (*15*,*19*,*20*). Pooled allantoic fluid was used for each study. A/Anhui/1/2013 (H7N9) used in these experiments was passaged 3 times in eggs from the original patient sample, and the sequence of the virus inoculum corresponded to Global Initiative on Sharing Avian Influenza Data accession no. EPI_ISL_138739. Experiments were performed under Biosafety Level 3+ containment in accord with the federal regulations (US Department of Agriculture 9 CFR 121 and 7 CFR 331, www.aphis.usda.gov/programs/ag_selectagent/downloads/FinalRule3-18-05.pdf).

### Animals

Commercially acquired zebra finches (*Taeniopygia guttata*), society finches (*Lonchura striata domestica*) and parakeets (*Melopsittacus undulates*) and wild-caught house sparrows (*Passer domesticus*), were quarantined for 1–3 weeks and displayed no signs of disease before the experiment. We serologically tested 3 or 4 sentinel birds of each species (excluding sparrows because of limited availability) for influenza antibodies (H3, H5, H7) by hemagglutination inhibition (HI) assay and found them to be antibody negative. Swabs taken on day 0 were negative for virus isolation in eggs. Food was provided ad libitum, and a minimum of 0.25 L of water was provided daily with a full change of water every 48 h. All birds within a given group shared the same water and food troughs. Groups of birds were inoculated intranasally, intraocularly, and orally with $10^5$ $\log_{10}$ 50% egg infectious dose ($EID_{50}$) of pooled allantoic fluid containing A/Anhui/1/2013 (H7N9) in 100 μL of phosphate-buffered saline. The inoculated animals were co-housed with 2 (parakeets) or 3 (finches, sparrows) naïve, direct-contact birds. Each bird's oropharynx and cloaca were swabbed every second day for 10 days. For each sample, virus was isolated and titrated in eggs in triplicate (3 eggs/sample, 100 μL each of 6 serial $\log_{10}$ dilutions) as described (*15*,*20*). All animal experiments were approved by the St. Jude Animal Care and Use Committee and complied with all applicable US regulations.

### Necropsy

At 3 days post inoculation (dpi), 2 finches from each group and 1 sparrow were euthanized for necropsy. Parakeets were excluded from euthanasia and subsequent necropsy because of limited numbers. To prevent cross-contamination, organs were harvested in the following order, and instruments were cleaned after each organ was sampled: brain, eye, lung, trachea, small intestine, and large intestine. Tissues were homogenized, and virus was isolated and titrated in eggs as described (*15*,*20*). Birds that were found dead underwent similar necropsy, but only brain, lung, and combined (small and large) intestinal tissue were collected (*15*,*19*,*20*).

### Serology

At 16 dpi, serum was collected from all surviving animals and tested by HI assay with homologous (A/Anhui/1/2013, H7N9) and heterologous (A/songbird/Hong Kong/SB102/2001, H3N8; A/Vietnam/1203/04, H5N1) viruses by using horse erythrocytes as described (*21*). An HI titer ≥20 was considered indicative of recent infection with A(H7N9) virus, whereas titers <20 were considered negative.

### Statistical Analysis

Mean infectious titers and serum antibody titers were compared using the 1-tailed Student *t* test in Excel (Microsoft, Redmond, WA) or GraphPad Prism v5 (La Jolla, CA, USA) software.

## Results

### Replication and Pathogenicity of A(H7N9) Virus

All inoculated birds shed virus, but shedding was confined to the oropharynx; no virus was isolated at any time from the cloaca. Shedding was highest in the 2 finch species at 2 dpi, and virus titers shed by these birds were 1.5–1.9 $\log_{10}$ higher than those from the sparrows or parakeets ($p<0.001$). Subsequently, society finches showed higher shedding than sparrows at 4 dpi ($p<0.001$), but the remaining groups did not differ in levels of virus shedding. Virus was shed for 6 days by finches and parakeets and for 4 days by sparrows, and >80% of the zebra finches and parakeets continued to shed virus at 6 dpi (Table 1). Virus had cleared in all inoculated animals by 8 dpi. One sparrow and 1 zebra finch were found dead at 3 and 6 dpi, respectively (Table 1), but only the sparrow had shown clinical signs of disease (lethargy; loose, discolored feces; ruffled feathers). Surviving inoculated birds were free of disease signs, although a

Table 1. Oropharyngeal and cloacal virus titers in birds inoculated with influenza A(H7N9) virus*

| Species | Titer from oropharyngeal swab† | | | | No. deaths‡ |
|---|---|---|---|---|---|
| | 2 dpi | 4 dpi | 6 dpi | 8 dpi | |
| Zebra finch | 4.8 ± 0.5 (7/7) | 3.8 ± 1.3 (5/5) | 2.9 ± 1.0 (5/5)§ | < | 1/5 |
| Society finch | 4.9 ± 0.5 (7/7) | 3.9 ± 0.7 (5/5) | 1.0 ± 0.0 (1/5) | < | 0/5 |
| Sparrow | 3.0 ± 0.5 (6/6) | 3.0 ± 0.7 (3/4) | < | < | 1/5 |
| Parakeet | 3.4 ± 0.5 (5/5) | 3.9 ± 1.6 (4/5) | 2.6 ± 0.1 (4/5) | < | 0/5 |

*dpi, days post inoculation; <, below the limit of detection (<0.75 $EID_{50}$/mL); $EID_{50}$, 50% egg infectious dose.
†$Log_{10}$ $EID_{50}$/mL. Data are the mean ± SD of positive samples (no. birds shedding virus/total no. sampled at the indicated time point). All cloacal samples were below the limit of detection at all time points.
‡Number of animals found dead out of the total for each group; excludes necropsied animals.
§Includes the animal found dead on this day.

slight decrease in food consumption and emptying of food troughs was observed at 6–9 dpi among zebra finches. In conclusion, all 4 species of small birds tested were susceptible to infection with A/Anhui/1/2013.

### Shedding of Virus into Water

Each day for 6 days, water was sampled from the communal trough shared by birds within each cage group, and virus was titrated in eggs. Virus was detected in all water troughs and on multiple days (Figure). Both finch species shed virus into the water on every postinoculation day studied, with the exception of 3 dpi in the zebra finches. Virus was not shed into the water until 3 dpi by the sparrows and parakeets. Zebra finches tended to shed more virus into the water than did sparrows or parakeets, and mean titers across of all sampled times differed significantly between these groups ($p<0.05$). However, the possibility that bird groups consumed different levels of water on any given day could not be normalized.

### Shedding by Direct Contacts

In the songbirds and parakeets, A/Anhui/1/13 virus was not highly transmissible to direct-contact animals. A single contact zebra finch showed trace amounts of virus at 2 and 4 dpi, and 2 sparrows showed trace amounts of virus at 4 dpi. Contact parakeets remained virus negative. In contrast, 1 contact society finch shed high titers of virus from 2 dpi ($10^{5.8}$ $\log_{10}$ $EID_{50}$/mL) through 8 dpi (Table 2). As with the inoculated animals, direct contacts shed virus only by the oropharyngeal route.

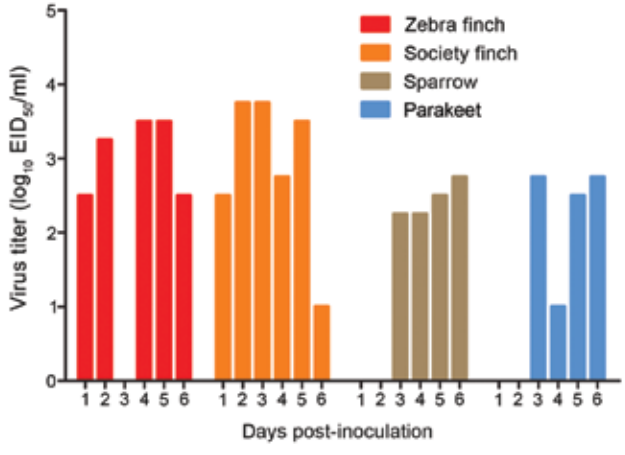


Figure. Virus shedding into water trough. A 500-μL sample of water was collected daily for 6 days, and virus was titrated in chicken eggs. The lower limit of detection was 0.75 50% egg infectious dose/mL.

### Isolation of Virus from Organs

Organs from inoculated birds were recovered 3 dpi, and virus was isolated and titered in chicken eggs. The sparrow that underwent necropsy showed trace virus only in the lungs (Table 3). Both finch species showed high virus titers in the trachea (4.5–4.6 $\log_{10}$ $EID_{50}$/mL). In the zebra finches, virus was observed only in the tracheas, consistent with swab findings, but 1 of 2 society finches showed trace amounts of virus in the brain and eye, whereas the other had trace amounts in the small and large intestine and high lung virus titer (5.8 $\log_{10}$ $EID_{50}$/mL) (Table 3). Two donor birds (one sparrow and one zebra finch) died during the experiment and underwent necropsy. No virus was isolated in any of the sparrow's organs. However, in the zebra finch, virus was detected in brain, lung, and intestine (2.5, 5.5, and 2.5 $\log_{10}$ $EID_{50}$/mL, respectively), suggesting that finches are vulnerable to extrapulmonary A(H7N9) virus infection (Table 3).

### Rates of Seroconversion

All surviving birds were tested for seroconversion by HI assay with serum collected at 16 dpi (Table 4). Among inoculated birds, 100% of society finches and sparrows seroconverted to homologous virus, as did 75% of zebra finches and 80% of parakeets. Mean HI titers in inoculated birds ranged from 4.8 to 6.9 $\log_2$ (HI 30–140) (Table 4). All contact zebra finches seroconverted, but only 1 of 3 society finches and 2 of 3 sparrows seroconverted. No seroconversion of contact parakeets was observed. Mean HI titers in contact animals that seroconverted were 4.3–6.3 $\log_2$ (HI 20–80). Mean titers were highest in society finches and lowest in parakeets, although they did not differ significantly in inoculated versus contact groups or according to species. HI titers to heterologous human subtype H5 and songbird subtype H3 viruses were negative (Table 4).

Table 2. Shedding of influenza A(H7N9) virus by direct contact among birds*

| Species | Titer from oropharyngeal swab† | | | |
|---|---|---|---|---|
| | 2 dpi | 4 dpi | 6 dpi | 8 dpi |
| Zebra finch | 1.0 ± 0.0 (1/3)‡ | 1.0 ± 0.0 (1/3) ‡ | < | < |
| Society finch | 5.8 ± 0.0 (1/3) | 3.5 ± 0.0 (1/3) | 2.3 ± 0.0 (1/3) | 2.8 ± 0.0 (1/3) |
| Sparrow | < | 1.9 ± 1.2 (2/3) | < | < |
| Parakeet | < | < | < | < |

*dpi, days post inoculation; <, below the limit of detection (<0.75 $EID_{50}$/mL); $EID_{50}$, 50% egg infectious dose.
†$Log_{10}$ $EID_{50}$/mL. Data are the mean ± SD of positive samples (no. birds shedding/total no. sampled at the indicated time point). All cloacal samples were below the limit of detection at all time points.
‡Sample contained trace amount of virus: 1 or 2 of 3 inoculated eggs was positive at the lowest serial dilution.

## Discussion

We assessed parakeets and 3 species of songbirds for their susceptibility to avian-origin A(H7N9) (A/Anhui/1/2013) virus and found that they were highly susceptible to infection with this isolate. Shedding was limited to the oropharynx, which may have reduced contact transmission; however, at most times sampled, the water troughs contained large amounts of virus. Furthermore, low-pathogenicity influenza viruses (those lacking a multibasic cleavage site in the hemagglutinin protein), such as the A(H7N9) isolate used here, remain stable in water longer than their highly pathogenic counterparts (*22*) and could therefore serve as an inoculum for cage mates. Despite little shedding in the contacts, seroconversion of at least 1 contact animal in each of the finch and sparrow groups indicates exposure with antigenic epitopes of the subtype H7 hemagglutinin. The minimal transmission to direct contacts observed in our study is consistent with previous observations of avian influenza virus infection of songbirds (*15*,*20*,*23*–*25*). The parakeets in particular showed no contact animal shedding or seroconversion, a phenomenon we have previously observed with an A(H3N8) isolate from a songbird (R.G. Webster et al., unpub. data). This observation may have implications for risk assessment of this species in the pet bird trade; however, the lack of proper influenza transmission data with this species in the literature warrants additional studies to confirm the validity of this observation. One direct-contact society finch shed virus equivalent to titers in inoculated birds and shed for a longer period, which suggests that efficient transmission and replication in contact animals, although rare, is possible. The ability of these small birds to harbor and shed A(H7N9) viruses, usually with few signs of illness, creates a substantial potential for transmission to humans, as well as to poultry and wild birds.

Interspecies transmission has not yet been investigated, and the extent to which A(H7N9)-infected finches, sparrows, and parakeets may transmit virus to other species, including through shared water sources, is unknown. Two host groups have the greatest potential interaction with small birds. The first is domesticated poultry, primarily chickens but also a wide variety of gallinaceous and game birds. The peridomestic nature of songbirds facilitates an interaction with poultry in large production facilities and in backyard farms (*14*,*15*). In these cases, they may share common food and water sources (*15*). The interspecies transmission of influenza from songbirds to poultry is not without precedent. Nestorowicz et al. proposed that highly virulent A(H7N7) isolates from starlings and chickens were closely related and indicated that the virus had been transmitted between these 2 species (*23*). Forrest et al. experimentally demonstrated waterborne transmission of highly pathogenic A(H5N1) virus from chickens to starlings (*15*), although the high death rate of the inoculated birds might have limited the degree of interspecies transmission. In the case of the low-virulence A(H7N9) viruses, most inoculated birds shed virus while remaining clinically healthy. The absence of illness and death in A(H7N9)-infected birds has implications for a greater quantity and duration of virus shedding into the environment, as well as higher activity levels and likelihood of interaction with other susceptible hosts. Preliminary data suggest that chickens and quail are highly susceptible to infection with this human A(H7N9) isolate and that the virus is readily transmitted by direct contact (*9*,*10*). The high titers recovered from the shared water troughs of all species tested in our study suggest that the virus could be transmitted to poultry and other birds through this route. Therefore, reduced interaction of domestic poultry with wild passerine birds is advisable, although this precaution might not be feasible in developing countries where numerous backyard farms lack biosecurity. Comprehensive biosecurity also is often lacking at large poultry farms in

Table 3. Influenza A(H7N9) virus replication in organs of inoculated birds*

| Species | Organ titer† | | | | | |
|---|---|---|---|---|---|---|
| | Brain | Eye | Trachea | Lung | Small intestine | Large Intestine |
| Zebra finch | < | < | 4.5 ± 0.0 (2/2) | < | < | < |
| Society finch | 2.5 ± 0.0 (1/2) | 1.0 ± 0.0 (1/2) | 4.6 ± 2.7 (2/2) | 5.8 ± 0.0 (1/2) | 1.0 ± 0.0 (1/2)‡ | 2.5 ± 0.0 (1/2) |
| Sparrow | < | < | < | 1.0 ± 0.0 (1/1)‡ | < | < |

*<, below the limit of detection (<0.75 $EID_{50}$/mL); $EID_{50}$, 50% egg infectious dose.
†$Log_{10}$ $EID_{50}$/mL. Data are the mean ± SD of positive (≥0.75 $EID_{50}$/mL) samples (no. birds shedding/total no. sampled at the indicated time point).
‡Sample contained trace amount of virus: 1 or 2 of 3 inoculated eggs was positive at the lowest serial dilution.

Table 4. Seroconversion of birds to influenza A(H7N9)*

| Species, exposure | HI titer† Baseline‡ | Homologous virus | Heterologous virus§ |
|---|---|---|---|
| Zebra finch | | | |
| Inoculated | < | 5.3 ± 1.0 (3/4) | < |
| Contact | < | 4.3 ± 0.0 (3/3) | < |
| Society finch | | | |
| Inoculated | < | 6.9 ± 0.9 (5/5) | < |
| Contact | < | 6.3 ± 0.0 (1/3) | < |
| Sparrow | | | |
| Inoculated | ND | 5.8 ± 0.6 (4/4) | < |
| Contact | ND | 4.3 ± 0.0 (2/3) | < |
| Parakeet | | | |
| Inoculated | < | 4.8 ± 0.6 (4/5) | < |
| Contact | < | < (0/2) | < |

*HI, hemagglutination inhibition; <, below the limit of detection (serum dilution<1:20); homologous virus: A/Anhui/1/2013(H7N9); heterologous viruses: A/Songbird/Hong Kong/SB102/2001 (H3N8) and A/Vietnam/1203/04 (H5N1); ND, not determined because of limited number of available birds; HA, hemagglutinin.
†Reciprocal value ($log_2$/50 μL) of the highest titer that inhibited 4 HA units of virus (no. seropositive animals/total no. sampled). Data are the mean ± SD of positive samples.
‡Baseline HI titers (3 birds/group) were obtained before virus challenge.
§HI titers to heterologous viruses were determined in the serum (16 dpi) from the same number of birds used to determine the HI titers to the homologous virus.

industrialized and developing countries and should be enhanced to limit the access of wild songbirds to the poultry's water and food sources.

A second host group with high potential to interact with songbirds and other small terrestrial birds are humans. Finches, sparrows, and parakeets are not only common in the wild but are popular pets worldwide (*26*). They are often sold in the live-bird markets of eastern Asia, where the risk for zoonotic influenza transmission of H7N9 and other influenza subtypes is already established (*26*,*27*). Such pet birds may be procured from the wild and may have been exposed to a variety of pathogens before entering the market chain (*9*). In China, the keeping of pet birds is associated with luck (*28*) and is common among elderly men, who often stroll through the parks with their caged birds (*28*,*29*). In caring for such pets, their owners could become infected by virus contaminated drinking water or from fomites on the feathers (deposited while bathing in water troughs or from saliva while preening) (*30*). This same demographic group (elderly men) experienced disproportionate rates of illness and death from A(H7N9) infection in China (*31*). A recent epidemiologic study by Rivers et al. concluded that the comparatively higher rates of infection among the elderly than among younger age groups cannot be entirely attributed to increased exposure to poultry. Furthermore, they assert that an "as-yet unknown epidemiological or immunological feature" may explain the high infection rates among older persons (*27*), which leaves open the contribution of alternate exposure sources, such as infected pet birds, as a possibility. Virus also could be transmitted to humans through religious ceremonies, such as the Buddhist practice of "merit release," in which a songbird is purchased, held to the face, kissed, and released (*25*). Simply owning a pet bird increased a household's rate of seroconversion during the 2003 A(H7N7) outbreak in the Netherlands (*32*). Although completely avoiding contact with pet birds during an avian influenza outbreak might not be feasible, surveillance of such species in the markets, and perhaps in the wild, would help to identify or rule out previously unsuspected hosts that might support or disseminate emerging viruses.

To expand on our findings, future studies should include the examination of genetic changes in the human A(H7N9) virus during replication and transmission in the songbirds and parakeets. The human isolate we used contained genetic markers (HA: S138A, G186V, and Q226L; PB2: E627K) indicating adaptation to mammals (*33*). Sequence analysis of virus shed by the small birds in our study might indicate which species would be most receptive to transmission. The loss of the markers of mammalian adaptation and reversion to an avian-like genotype might predispose the virus to transmission from the small birds to poultry and other birds, rather than to humans. Additionally, studies that assess the sharing of housing, water, and food by inoculated songbirds and poultry (particularly chickens or quail) would shed light on the interspecies transmission potential of A(H7N9) viruses.

Our demonstratration that parakeets and multiple species of songbirds are susceptible to influenza A(H7N9) virus isolated from humans during the recent outbreak in China further supports the possible contribution of songbirds and parakeets to the ecology, maintenance, and transmission of novel A(H7N9) viruses. Finally, they lead us to propose that finches, sparrows, and parakeets may be intermediate hosts and sources of A(H7N9) viruses and that their frequent interaction with wild birds, domestic poultry, and humans renders them a particular risk factor in the emergence and transmission of novel influenza strains.

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interests include the host response to influenza viruses, emergence of novel influenza variants, and zoonotic transmission of influenza between species.

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# Hantavirus Infections among Overnight Visitors to Yosemite National Park, California, USA, 2012

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In summer 2012, an outbreak of hantavirus infections occurred among overnight visitors to Yosemite National Park in California, USA. An investigation encompassing clinical, epidemiologic, laboratory, and environmental factors identified 10 cases among residents of 3 states. Eight case-patients experienced hantavirus pulmonary syndrome, of whom 5 required intensive care with ventilatory support and 3 died. Staying overnight in a signature tent cabin (9 case-patients) was significantly associated with becoming infected with hantavirus (p<0.001). Rodent nests and tunnels were observed in the foam insulation of the cabin walls. Rodent trapping in the implicated area resulted in high trap success rate (51%), and antibodies reactive to Sin Nombre virus were detected in 10 (14%) of 73 captured deer mice. All signature tent cabins were closed and subsequently dismantled. Continuous public awareness and rodent control and exclusion are key measures in minimizing the risk for hantavirus infection in areas inhabited by deer mice.

Hantavirus pulmonary syndrome (HPS) is an acute viral disease, characterized by a nonspecific febrile illness followed by severe noncardiogenic pulmonary edema and cardiogenic shock. It is also referred to as hantavirus cardiopulmonary syndrome. Approximately 36% of reported HPS cases in the United States are fatal (*1*). HPS was first recognized in 1993 when an outbreak of unexplained respiratory deaths occurred among otherwise healthy adults in the southwestern United States (*2*–*5*). A previously unknown hantavirus, Sin Nombre virus (SNV), was identified as the etiologic agent (*6*,*7*). Deer mice (*Peromyscus maniculatus*) are the reservoir for SNV (*8*), which infected mice shed in their urine, saliva, and feces. Humans are exposed chiefly by inhaling aerosolized excreta. Activities associated with increased risk for HPS include occupying or cleaning rodent-infested buildings, sweeping or disturbing rodent excreta or nests, sleeping on the ground, and handling mice without gloves (*9*–*11*). Person-to-person transmission of SNV has not been documented (*12*).

During 1993–2011, a total of 587 cases of HPS were confirmed in the United States, primarily in western states (Centers for Disease Control and Prevention [CDC]/Viral Special Pathogens Branch, unpub. data). Although household clusters of HPS have been reported (*13*,*14*), most HPS cases occur as single, sporadic disease incidents. In August 2012, the California Department of Public Health (CDPH) confirmed HPS in 2 California residents, both of whom had visited Yosemite National Park (Yosemite) in June 2012 and had lodged in so-called signature tent cabins (which differ from regular tent cabins by having an interior wall and roof consisting of drywall with a layer of foam insulation between the drywall and exterior canvas) in the Curry Village area of Yosemite Valley. Because of the common travel history of these otherwise nonepidemiologically unrelated patients, CDPH, in collaboration with CDC, the National Park Service (NPS) Office of Public Health, and others, initiated an investigation of a possible HPS outbreak associated with Yosemite. This report summarizes the clinical, epidemiologic, laboratory, and environmental findings of the investigation.

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## Methods

### Epidemiologic and Laboratory Investigation

We defined a case-patient as a person who had 1) illness onset June 1–October 31, 2012, clinically compatible with hantavirus disease; 2) laboratory confirmation of SNV infection by detection of IgM against SNV or increasing titers of IgG in serum, SNV-specific antigen shown by immunohistochemical test (IHC), or SNV-specific nucleic acid sequences by reverse transcription PCR (RT-PCR); and 3) a history of visiting Yosemite during the 6 weeks preceding illness onset. HPS was defined as fever ≥38.3°C with bilateral diffuse interstitial edema and respiratory compromise within 72 hours of hospitalization, occurring in a previously healthy person, or an unexplained respiratory illness resulting in death, for which an autopsy demonstrated noncardiogenic pulmonary edema without an identifiable cause (*15*).

Finding case-patients was facilitated through health alerts issued by CDPH, NPS, and CDC to health care providers, advising them to contact state or local public health officials to obtain confirmatory testing of patients with suspected HPS who had visited Yosemite. Commercial diagnostic laboratories that detected hantavirus antibody in submitted serum specimens were asked to notify and forward specimens to state public health laboratories or CDC for confirmatory testing. State health departments that identified the death of a previously healthy person who had a history of travel to Yosemite were asked to collect tissue specimens for confirmatory testing by CDC. CDPH and NPS issued media releases recommending that persons who experienced febrile illness after travel to Yosemite consult with their health care provider about possible hantavirus infection. Notifications were sent to all visitors registered as overnight guests at Yosemite during June 1–September 12, 2012, advising them to seek medical attention if they experienced symptoms of hantavirus infection.

Acute- or convalescent-phase serum specimens were tested at CDPH's Viral and Rickettsial Diseases Laboratory or CDC's Viral Special Pathogens Laboratory for IgM and IgG against SNV by using the same ELISA (*7*). Four-fold dilutions were performed (1:100–1:6400), and titers ≥400 were considered positive. Formalin-fixed tissues from patients who died were tested for the presence of hantavirus antigen by IHC at CDC's Infectious Diseases Pathology Branch (*16*). A nested RT-PCR (*17*) was performed by using RNA extracted from frozen tissue. A TaqMan assay (Roche Molecular Systems, Inc., Pleasanton, CA, USA), targeting the small (S) segment RNA from North American hantaviruses, was used on RNA samples extracted from fixed tissues. This assay was used following standard protocols (SuperScript III Platinum One-Step qRT-PCR Kit w/ROX; Invitrogen, Carlsbad, CA, USA) with the following primers and probes: 143F 5′-TGGACCCIGATGAYGTTAACAA-3′, 303R 5′-ATCAGGITCAAKCCCIGTTGG-3′, and NA-181P 5′-FAM-AGACGGGCIGCTGTGTCTGCATT-BQH-3′. Medical records for laboratory-confirmed HPS patients were reviewed for relevant clinical information.

Patients with confirmed cases (or a proxy family member if the patient was deceased) and their non-ill travel companions who had shared lodging with the patient were interviewed by telephone, using a standardized questionnaire. One non-ill companion was interviewed for each patient identified. By using closed and open-ended questions, patients and non-ill companions were asked about demographic characteristics, illness history, travel, and other activities in the weeks preceding illness onset. Participants were asked about the type of lodging and activities they experienced during their Yosemite visit, particularly actions (e.g., cleaning or sweeping), observations (e.g., mouse droppings), and behavior (e.g., sleeping on the floor) potentially associated with exposure to rodents and SNV. Data from questionnaires were maintained in a spreadsheet by using standard software (Excel; Microsoft, Redmond, WA, USA). The incidence of SNV infection among guests staying in a signature tent cabin was compared with that of guests staying in other lodgings in Curry Village, according to Yosemite guest registry records for June 1–August 28, 2012. A difference was considered statistically significant if $p \leq 0.05$.

### Environmental Investigation

Yosemite lodgings where patients had stayed and other nearby buildings were visually evaluated for evidence of active or recent rodent activity (e.g., nesting materials, feces, or urine stains) as well as structural features that might allow rodent entry. Rodent populations in and around Yosemite guest lodgings were live-trapped for evaluation. Sherman live-traps (H.B. Sherman Traps, Tallahassee, FL, USA) were baited with rolled oats and placed overnight inside and outside buildings. Rodent abundance was estimated by trap success, defined as the ratio of captured mice to the total number of traps set. Captured rodents were anesthetized, euthanized, measured, and identified to species. A minimum of 11 mL of blood was collected from the retrobulbar sinus of each mouse and sent to CDPH for ELISA testing for IgG against SNV.

## Results

### Laboratory, Clinical, and Epidemiologic Findings

Ten case-patients were identified among persons who had visited Yosemite overnight during summer 2012. The median age of the 10 patients was 44.5 years (range 12–56 years). Eight were residents of California, 1 of Pennsylvania,

and 1 of West Virginia. Infection was confirmed by serologic testing for 8 patients and by IHC and RT-PCR for 2 patients. A 540-bp fragment amplified from fresh frozen tissues of 1 case-patient who died had high homology (>90%–99% identity) with the known SNV isolates Convict Creek, NM R11, and NM H10 (GenBank accession nos. AF425256.1, L37902.1, and L37901.1, respectively).

Salient case-patient clinical characteristics are displayed in the Table. Eight patients had respiratory signs or findings compatible with HPS, 3 of whom died. Seven HPS case-patients were hospitalized for a median of 7 days (range 2–30 days); 5 required intensive-level care with ventilatory support.

The illnesses of 2 case-patients did not fulfill the complete HPS definition. One patient had been examined in an emergency department for fever, cough, and shortness of breath and was discharged and recovered. That patient's chest radiograph and computed tomographic scan revealed no evidence of pulmonary infiltrates or edema. The second patient did not report fever or pulmonary symptoms but had severe headache, nausea, vomiting, and diarrhea. Both patients were tested for SNV retrospectively after they heard about the Yosemite outbreak in the news and had detectable antibodies against SNV.

The onset of illness for the 10 case-patients occurred from July 2 through August 16, 2012 (Figure 1). They had stayed overnight at Yosemite during June 3–July 23, 2012; the median incubation period was 30.5 days (range 20–49 days). From interviews with case-patients (or their proxy and non-ill companions), we found that case-patients reported engaging in activities and behavior similar to those of non-ill companions while at Yosemite, and no single activity typically associated with risk for HPS was common among patients.

Table. Clinical characteristics of 10 hantavirus case-patients who were exposed at Yosemite National Park, 2012

| Patient characteristic | No. affected |
|---|---|
| Male sex | 6 |
| Illness clinically compatible with hantavirus pulmonary syndrome | 8 |
| Hospitalized | 7 |
| Intubated | 5 |
| Died | 3 |
| Pulmonary edema on chest radiographs | 7* |
| Thrombocytopenia (<150,000/mm$^3$) | 8* |
| Elevated hematocrit (>50%) | 1* |
| Elevated creatinine (>1.3 mg/dL) | 4* |

*Diagnostic tests were not performed for 2 case-patients, 1 with only mild symptoms and 1 who died.

Nine case-patients had lodged 1–6 nights in a signature tent cabin in the Boystown area of Curry Village. One of these patients stayed in the same cabin in which another patient had lodged 13 days earlier. Staying overnight in a signature tent cabin was significantly associated with SNV infection: 9 of 10,193 guests who registered to stay in a signature tent cabin during June 1–August 28, 2012, had confirmed SNV infection, compared with 0 of 40,288 registered guests of other cabins in Curry Village during the same period ($p<0.001$). One case-patient had not lodged in Curry Village but had stayed in 4 different regular tent cabins in the Tuolumne Meadows area of Yosemite, ≈16 miles northeast of Curry Village and >4,700 feet higher in elevation.

Of all guests who registered to stay in a signature tent cabin during this time, ≈50% were from California, 30% were from other states, and 20% were from other countries. No SNV infections were reported among international visitors to Yosemite. No hantavirus infections were reported among ≈2,800 NPS and contract employees who worked in Yosemite during the outbreak period.

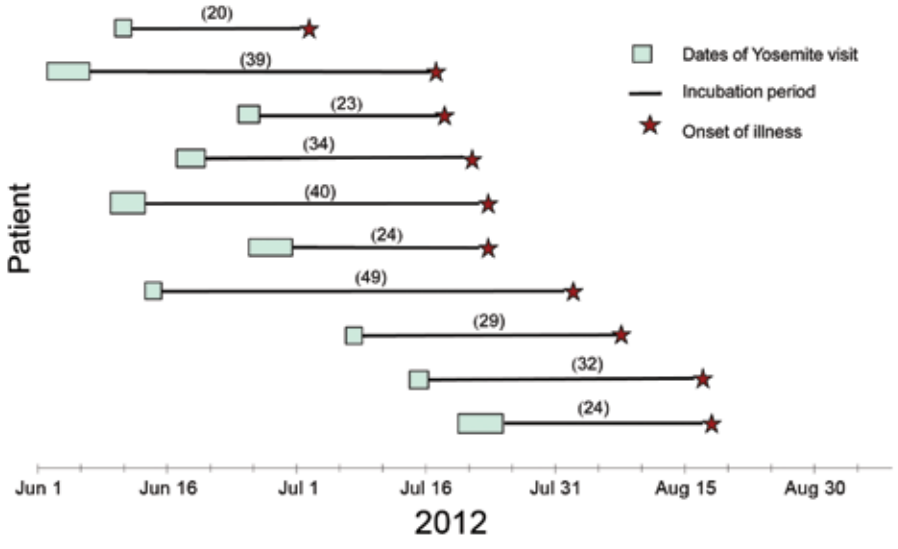


Figure 1. Dates of stay in Yosemite National Park, incubation periods, and dates of illness onset for 10 case-patients, 2012. Incubation period calculated as days from last date of Yosemite stay to first date of illness.

### Environmental Findings

Guest lodgings in Curry Village, at an elevation of ≈4,000 feet above sea level, consisted of 319 regular tent cabins, 91 signature tent cabins, 18 standard hotel rooms, and 70 hard-sided wood cabins on ≈23 acres. All signature tent cabins were located in the single 2.5-acre Boystown part of Curry Village. Both regular tent cabins and signature tent cabins are constructed of an exterior heavy canvas affixed over a wood frame elevated off the ground on a wood base (Figures 2, panels A, B). The distinct characteristics of signature tent cabins are shown in Figure 2, panel C, and Figure 3, panel B.

In August 2012, direct (e.g., feces) and indirect (e.g., gnaw holes) evidence of rodent activity was observed in both signature and regular tent cabins. Opportunities for mouse entry were also observed for both types of cabins. Regular tent cabins evaluated often had gaps between the door and threshold and gaps between the canvas and the frame or foundation. Most signature tent cabins evaluated had gaps between the door and threshold, gaps between the outer canvas tent and inner insulated wall, and holes in the exterior walls or floors, particularly around conduits for heaters. In late August 2012, when the cabins were being inspected and retrofitted to close these gaps, evidence of active rodent infestation (tunneling and nesting and live mice) was observed in the wall foam insulation of most signature tent cabins (Figure 4).

In the Tuolumne Meadows area, ≈8,725 feet above sea level, the regular tent cabins were similar to those in Curry Village: heavy canvas was affixed over a wood frame with either a wood or a cement and stone foundation. Structural points of potential rodent entry were similar to those observed in Curry Village.

Rodent surveillance was conducted over 2 successive nights each in August and September 2012. On August 21–22, a total of 185 traps were placed around the signature tent cabins and other buildings in Curry Village. A total of 95 *Peromyscus* mice (trap success 51%) were collected: 73 *P. maniculatus* mice, 16 *P. boylii* mice, and 6 mice of *Peromyscus* species that could not be identified to species because raccoons had preyed on the mice in the traps. Serum antibodies against SNV were detected in 10 (14%) of 73 *P. maniculatus* and 0 of 16 *P. boylii* mice. On September 4–5, a total of 133 traps were placed in Curry Village, primarily in Boystown, and 89 traps were placed in Tuolumne Meadows. In Curry Village, 10 *P. maniculatus* mice and 9 *P. boylii* mice were collected (trap success 14%); no SNV serum antibodies were detected. In Tuolumne Meadows, 39 *Peromyscus* mice (all *P. maniculatus*), were collected (trap success 44%); SNV serum antibodies were detected in 2 (5%).

### Outreach and Prevention Efforts

During August 27–September 17, 2012, Yosemite contacted ≈10,000 guests who had stayed in signature tent cabins, ≈30,000 who had stayed in regular tent cabins, and ≈230,000 guests who had stayed in other park lodging, representing visitors from ≈77 different countries. The Yosemite public hotline received >4,800 calls. Hantavirus


Figure 2. Regular and signature tent cabins, Yosemite National Park, summer, 2012. A) Outside view of a regular tent cabin. B). Outside view of a signature tent cabin. C) Inner layer of foam insulation under the canvas of a signature tent cabin.


Figure 3. Inside view of regular and signature tent cabins, Yosemite National Park, summer 2012. A) Regular tent cabin showing canvas affixed over a wood frame. B) Signature tent cabin showing drywall between the exterior canvas and the interior living space.

educational materials were handed to every park visitor, and educational messages were posted in public areas, in lodgings, and on the park Internet site. Additional hantavirus awareness and prevention training sessions were provided to employees and volunteers within the park and NPS-wide. CDC developed an Internet page specific to the outbreak and added staff to its toll-free hantavirus hotline.

On August 28, 2012, Yosemite closed all 91 signature tent cabins indefinitely when it was confirmed that 4 of the 6 patients identified at that time had lodged in 1 of the cabins and when mouse infestations in the cabin walls were noted. The cabins were subsequently dismantled. Approximately 1,300 buildings throughout the park were inspected, and rodent exclusion was performed where needed. Enhanced housekeeping, inspection, and documentation and response protocols were implemented. Additionally, park management began a rodent population surveillance program and implemented trapping in highly developed areas of Yosemite Valley. NPS-wide efforts include enhanced education and training opportunities, integration of public health review into building design and modification reviews, and enhanced guidelines for routine rodent exclusion inspections in all park structures.

## Discussion

The 2012 outbreak of hantavirus infections among Yosemite visitors was the largest recognized outbreak of SNV infections in the United States since the Four Corners outbreak in 1993. Ten persons had laboratory-confirmed SNV infection after lodging in Yosemite during June–July 2012. Eight had HPS and 3 died. In 1 patient, HPS had an incubation period of 7 weeks, longer than the 6-week upper range typically reported for HPS (*5*,*18*).

Staying overnight in a signature tent cabin in Yosemite's Curry Village during June–August 2012 was significantly associated with SNV infection. While staying at Yosemite, case-patients had engaged in activities and behavior that were similar to those of their non-ill travel companions, and no common behavioral risk was identified. Despite extensive notification of all registered guests during the outbreak period, no cases were identified among persons who had lodged in regular tent cabins or elsewhere in Curry Village. No cases were identified among park employees, some of whom routinely entered signature tent cabins for housekeeping duties. No cases were identified among park visitors who had not stayed overnight.

This rare hantavirus outbreak was unusual because it was associated with exposure to rodents in a specific type of housing. Yosemite's signature tent cabins were unique to the Boystown area in Curry Village and were not located elsewhere in Yosemite or in the NPS system. They were constructed in 2009 with dry wall and foam insulation to provide lodging for guests during winter. The presence of active deer mouse infestations within the insulated spaces of the cabins likely increased the risk for SNV exposure for persons staying overnight in these cabins. Although lodging in a signature tent cabin was a statistically significant risk factor for SNV infection, the overall incidence was small, occurring in ≈1 of 1,000 guests.

The 51% trap success for *Peromyscus* mice in Curry Village in August 2012 was substantially higher than the 11% (58 of 538) trap success in Yosemite Valley, including Curry Village, in April 1995 (CDPH, unpub. data) and 13%–21% trap success at ecologically comparable US Forest Service facilities in California in 2004–2005 (*19*). These data indicate that a robust population of deer mice

Figure 4. Damage from rodents tunneling in the foam insulation of a signature tent cabin, Yosemite National Park, summer 2012.

was active at Curry Village in summer 2012. This population, however, appeared to be substantially lower in September (trap success 14%), after the signature tent cabins were closed in late August and rodent control measures were implemented. The presence of SNV (14% seroprevalence) among these deer mice was comparable to the 16% seroprevalence reported previously in deer mice trapped at similar elevations in California (*19*).

One case-patient had not stayed in a signature tent cabin or in Curry Village but had lodged in regular tent cabins in the Tuolumne Meadows area. Although this patient had visited Yosemite during the same period, his illness was unrelated epidemiologically to the signature tent cabin exposures linked to the other patients and represented a sporadic case of hantavirus infection. Two previous cases of HPS were identified in 2000 and 2010 among California residents who had visited the Tuolumne Meadows area during their illness incubation periods. Rodent surveillance conducted in September 2007 and September 2008 in Tuolumne Meadows documented *Peromyscus* mice trap successes of 14% and 17%—all *P. maniculatus* mice (CDPH, unpub. data). The 44% trap success in September 2012 indicated that the deer mouse population there was more robust than in previous years. However, SNV serum antibodies had been noted in ≈22% of deer mice in Tuolumne Meadows in 2007–2008 (CDPH, unpub. data), an appreciably higher percentage than the 5% observed during our investigation.

The 2012 Yosemite outbreak differed from the 1993 Four Corners outbreak in certain respects. The Yosemite outbreak occurred over a much shorter period; Yosemite patients were exposed and experienced illness over 1.5 months, whereas cases from the Four Corners outbreak occurred over 7 months (*2*–*5*). Also, Yosemite patients' exposures occurred in a smaller geographic area and in a national park recreational area rather than private residential settings.

No specific treatment exists for HPS, but prompt recognition of symptoms and early initiation of supportive care can reduce death rates. In addition, extracorporeal membrane oxygenation might be helpful for infected patients who experience severe cardiopulmonary failure (*20*). Although HPS is rare and distinguishing its prodromal symptoms from other nonspecific viral syndromes is difficult, clinicians should consider HPS for all persons with febrile illness, sudden onset of respiratory symptoms, and progressive thrombocytopenia who have a history of possible rodent exposure. HPS remains a reportable disease in the United States, and all suspected cases should continue to be reported to the respective local or state health department for notification and confirmatory testing. Obtaining patient's thorough travel history, which includes potential rodent exposures in rural settings (e.g., camping or opening seasonally closed buildings), can be useful for ascertaining the possibility of hantavirus infection.

Two case-patients in our study had milder disease that did not progress to HPS. Mild and subclinical illnesses are believed to represent a minor proportion of SNV infections (*21*,*22*). These 2 milder cases probably would not have been recognized if not for the publicity generated about the outbreak. A diagnosis of hantavirus infection was considered and pursued for both patients only after recovery and because of their report of having visited Yosemite during the weeks preceding their illnesses. These 2 cases demonstrate that mild SNV infections might be underdiagnosed, and thus, the 10 clinical cases identified in this investigation might underestimate the true incidence of SNV infection in this outbreak. Furthermore, media attention regarding this outbreak also led to the identification of 2 fatal cases; at the time of death for both patients, HPS was not suspected, and

diagnostic testing was pursued only after media reports led clinicians to reconsider the cause of death.

The investigation possibly did not identify all cases of SNV infection among visitors to Yosemite during summer 2012. In addition, Yosemite visitors who had already recovered from a febrile illness at the time of notification might have been less motivated to again seek medical attention and be tested for SNV. Because HPS is a rare disease, hospitalized patients with unexplained acute respiratory distress syndrome might not have had HPS included in their differential diagnosis and therefore were not tested for hantavirus infection.

The observations at Curry Village made during the environmental investigation might not reflect conditions existing earlier, when the patients were exposed. The first environmental assessment was not conducted until ≈2 months after the earliest HPS patient had visited Yosemite. Nevertheless, the robust rodent population indicated by the high trap success in August 2012 and extensive evidence of deer mouse infestation of the signature tent cabins indicated that opportunity for exposure of guests to rodent excreta was likely high when the patients were staying there.

Yosemite management, in concert with local, state, and federal public health officials, took multiple actions to reduce the risk for hantavirus transmission to park visitors. Closing the signature tent cabins indefinitely, even before all patients were identified and all data were collected, was judicious and prevented more guests from being exposed. Regular tent cabins that replaced the dismantled signature tent cabins were designed and constructed with particular attention to rodent proofing. Continuous public awareness and rodent control and exclusion are key measures for minimizing the risk for hantavirus infection in areas inhabited by deer mice.

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# *Neisseria meningitidis* Serogroup W, Burkina Faso, 2012

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In 2010, Burkina Faso became the first country to introduce meningococcal serogroup A conjugate vaccine (PsA-TT). During 2012, Burkina Faso reported increases in *Neisseria meningitidis* serogroup W, raising questions about whether these cases were a natural increase in disease or resulted from serogroup replacement after PsA-TT introduction. We analyzed national surveillance data to describe the epidemiology of serogroup W and genotyped 61 serogroup W isolates. In 2012, a total of 5,807 meningitis cases were reported through enhanced surveillance, of which 2,353 (41%) were laboratory confirmed. The predominant organism identified was *N. meningitidis* serogroup W (62%), and all serogroup W isolates characterized belonged to clonal complex 11. Although additional years of data are needed before we can understand the epidemiology of serogroup W after PsA–TT introduction, these data suggest that serogroup W will remain a major cause of sporadic disease and has epidemic potential, underscoring the need to maintain high-quality case-based meningitis surveillance after PsA–TT introduction.

In Burkina Faso, which lies within the meningitis belt of sub-Saharan Africa, rates of endemic meningitis are high, seasonal epidemics occur each year, and explosive epidemics occur every 5–12 years (*1*,*2*). Historically, these epidemics have been caused primarily by *Neisseria meningitidis* serogroup A (*3*,*4*). During late 2010, Burkina Faso became the first country to introduce a novel meningococcal serogroup A polysaccharide–tetanus toxoid conjugate vaccine (PsA-TT, MenAfriVac (Serum Institute of India Ltd, Pune, India). More than 11 million persons were vaccinated during ≈10 days in the eligible population of persons aged 1–29 years (*4*,*5*). Early evidence suggests that this aggressive strategy substantially reduced the rate of meningitis among persons in these age groups and in the general population because of high coverage and herd immunity (*5*). Since 2010, nine countries have implemented PsA-TT; the 100 millionth dose was given in December 2012.

Although *N. meningitidis* serogroup A has virtually disappeared in countries that have implemented nationwide vaccination campaigns, other serogroups, including *N. meningitidis* serogroup W and serogroup X, have the potential to cause epidemics. Sporadic disease and localized epidemics caused by serogroup X have been well described, but the potential of serogroup X to cause large epidemics remains unclear (*6*). During 2000 and 2001, serogroup W was associated with outbreaks in pilgrims to the Hajj and was followed by several clusters of cases worldwide (*7*–*9*). The largest reported outbreak caused by serogroup W occurred in Burkina Faso in 2002 and comprised ≈13,000 suspected cases (*10*). Sporadic cases and smaller outbreaks caused by serogroup W have continued to occur and have been described as occurring in several meningitis belt countries, including Burkina Faso (*11*–*15*). During 2012, Burkina Faso reported an increase in cases of serogroup W, raising questions about whether the increase resulted from a natural increase in disease or resulted from serogroup replacement after PsA-TT introduction. We analyzed national surveillance data and isolates from Burkina

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Faso to describe the epidemiology of serogroup W during 2012 and to assess changes and trends in serogroup W epidemiology around PsA-TT introduction.

## Methods

Burkina Faso has 2 complementary systems of population-based meningitis surveillance (*5*). Surveillance for reportable diseases is conducted by the Télégramme Lettre Official Hebdomadaire (TLOH), to which district-level aggregate reports of clinically defined meningitis cases and meningitis-related deaths are transmitted weekly. Enhanced surveillance for Maladies à Potentiel Epidémique (MPE) collects case-level demographic information and results of cerebrospinal fluid (CSF) examination and laboratory testing. For this analysis, we used data from TLOH for 2007–2012 and MPE enhanced surveillance data for 2010–2012. All 63 districts reported suspected meningitis cases to both surveillance systems during 2010–2012. Aggregate laboratory testing results for the 2007–2009 meningitis seasons and enhanced surveillance data for the 2002 meningitis season also were analyzed.

We used a modified case definition to classify cases. Suspected cases of meningitis were defined as sudden onset of fever with a stiff neck or, in infants, a bulging fontanelle. Probable bacterial meningitis was defined as a suspected case for which gram-stained CSF was positive (gram-negative diplococci, gram-positive diplococci, or gram-negative bacilli); probable meningococcal meningitis cases are limited to those with gram-negative diplococci. A confirmed case of meningitis was defined as a suspected or probable case for which *N. meningitidis*, *Haemophilus influenzae* type b or *Streptococcus pneumoniae* was isolated in culture from blood or CSF, or antigen was detected in CSF by latex agglutination or PCR. Latex agglutination–positive cases that were later negative by PCR or culture were reclassified as suspected cases.

Beginning in 2010, real-time PCR capacity was implemented at the national reference laboratory, and detection of *N. meningitidis*, *H. influenzae* type b, or *S. pneumoniae* genetic material by PCR was deemed confirmatory (*16*,*17*). Serogroup was determined by antigen detection or by PCR, with PCR deemed definitive. Because serogroup Y is seen infrequently as a cause of disease in Burkina Faso, all specimens positive for serogroup Y/serogroup W by latex agglutination and not tested by PCR or culture were assumed to be serogroup W.

The meningitis season in Burkina Faso is limited to epidemiologic weeks 1–24. The pre–PsA-TT implementation period was defined as January 1, 2007–December 31, 2010, and the post–PsA-TT implementation period as January 1, 2011–December 31, 2012. Incidence rates were cumulative yearly incidence per 100,000 population and were calculated with national, regional, and district population estimates from the Institut National de la Statistique et de la Démographie. Districts crossed the World Health Organization weekly epidemic threshold when the weekly incidence rate exceeded 10 suspected cases per 100,000 population (*18*).

Multilocus sequence typing, porin A (PorA), and ferric enterobacin transport (FetA) genotyping was performed on 61 serogroup W isolates (6 from 2011 and 55 from 2012). Multilocus sequence typing was performed by sequencing the internal fragment of 7 housekeeping genes (*19*). PorA and FetA genotyping was performed by sequencing the variable regions (VRs) of *porA* (VR1 and VR2) and the VR of *fetA*. All generated sequencing data were analyzed by using the Web-based software MGIP (http://mgip.biology.gatech.edu/home.php). Molecular types (sequence type [ST], clonal complex [cc], PorA, and FetA) were determined by searching the PubMLST database (http://pubmlst.org). Strain genotype was defined as ST(cc):PorA:FetA.

## Results

During January 1–December 31, 2012, a total of 7,022 suspected meningitis cases (739 [11%] fatal) were reported through TLOH in Burkina Faso, corresponding to an incidence of 42.0 cases per 100,000 population. A total of 5,807 cases were reported through MPE case-based meningitis surveillance; 2,353 (41%) were confirmed, 415 (7%) were probable, and 3,039 (52%) were suspected meningitis cases. Of the 2,353 confirmed cases, 591 (25%) were confirmed by culture, 1,741 (74%) by PCR, and 21 (<1%) by latex agglutination. The predominant organism identified was *N. meningitidis* serogroup W (Table 1). More meningitis cases were reported during the 2012 than the 2011 meningitis season, and a higher proportion of cases were caused by serogroup W in 2012 than in 2011 (Figure 1).

Of persons with *N. meningitidis* serogroup W infection during 2012, a total of 11% were <1 year of age, 29% were 1–4 years, 29% were 5–9 years, 16% were 10–14 years, and 15% were ≥15 years. This distribution is similar to that

Table 1. Laboratory-confirmed bacterial meningitis cases, Burkina Faso, 2012*

| Bacterium, serogroup | Culture or PCR | Latex agglutination† | Total |
|---|---|---|---|
| *Neisseria meningitidis* | | | |
| A | 0 | 0 | 0 |
| W | 1,438 | 13 | 1,451 |
| X | 207 | NA | 207 |
| Y | 4 | 0 | 4 |
| C | 1 | 0 | 1 |
| Indeterminate | 125 | 0 | 125 |
| *Streptococcus pneumoniae* | 527 | 7 | 534 |
| *Haemophilus influenzae* type b | 30 | 1 | 31 |
| Total | 2,332 | 21 | 2,353 |

*Data are from Maladies à Potentiel Epidémique case-based surveillance. NA, not applicable.
†Excludes 62 latex agglutination–positive cases that were later ruled out by PCR and/or culture.

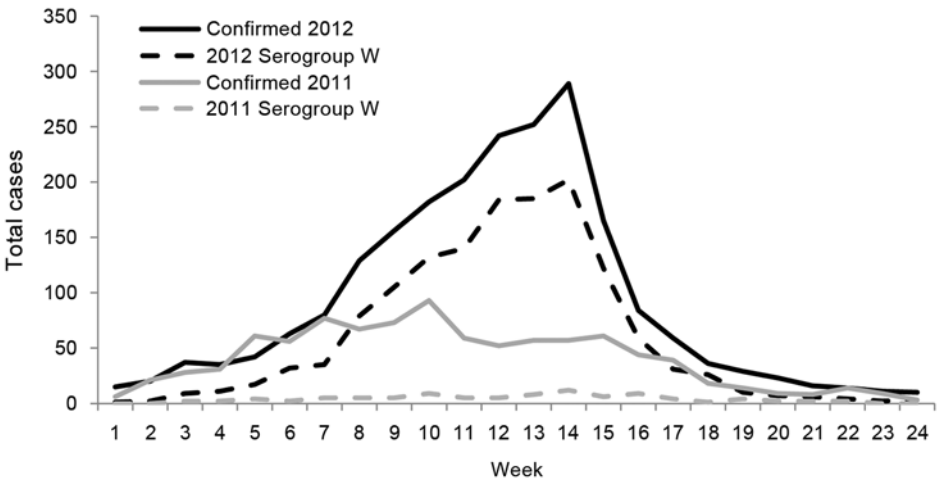


Figure 1. Laboratory-confirmed bacterial meningitis cases and *Neisseria meningitidis* serogroup W cases, Burkina Faso, 2011 and 2012 meningitis seasons. Data source: Maladies à Potentiel Epidémique (case-based surveillance).

for serogroup W cases during the 2002 serogroup W epidemic, according to enhanced surveillance data (serogroup W: 16% persons were <1 year of age, 28% were1–4 years, 31% were 5–9 years, 8% were 10–14 years, and 17% were ≥15 years). The incidence of laboratory-confirmed serogroup W was 8.7 cases per 100,000 population in 2012, compared with 0.7 per 100,000 in 2011. During 2007–2010, when <15% of cases were laboratory confirmed, the incidence of serogroup W was <0.1 cases per 100,000 population (Table 2).

The incidence of serogroup W varied by district, from 0.0 to 40.7 cases per 100,000 population (median 6.1/100,000) in 2012. By week 24, thirteen districts had crossed the weekly epidemic threshold of 10 suspected cases per 100,000 population (selected districts, Figure 2); *N. meningitidis* serogroup W was the predominant serogroup identified in 12 of these 13 districts (*N. meningitidis* serogroup X was the predominant organism identified in the 13th district). In no district did the cumulative incidence rate reach >100 cases per 100,000 population, the established definition of an epidemic, during 2012 (*20*). Three districts had reactive vaccination campaigns that used quadrivalent (A, C, W, Y) polysaccharide vaccine; in 2 of these districts, the number of cases crossed the weekly epidemic threshold. These districts were 2 of the last to cross the weekly epidemic threshold in 2012 during weeks 14 and 15. Cumulative incidence of serogroup W cases in these districts was 11.0 and 23.2 cases per 100,000 population, respectively.

We characterized 61 serogroup W isolates: 6 from 2011 and 55 from 2012, all belonging to cc11. The predominant genotype detected was ST-11(cc11):P1.5,2:F1–1, which accounted for 90% (55/61) of the total isolates. The remaining isolates differed only in the FetA and ST (ST-11[cc11]:P1.5,2:F6–3, 2/61 [3%]; ST-2961[cc11]:P1.5,2:F1–1, 2/61 [3%)]; and ST-9766[cc11]:P1.5,2:F1–1, 2/61 [3%]. Three STs were detected: ST-11 (57/61 [93%]), ST-2961 (2/61 [3%]), and ST-9766 (2/61 [3%]).

## Discussion

In 2012, the second year after PsA-TT introduction in Burkina Faso, the continued absence of serogroup A demonstrated the effect of this enormously successful vaccination campaign. *N. meningitidis* serogroup W was the predominant organism causing meningitis during the 2012 epidemic season. However, rates of serogroup W disease were substantially lower in 2012 (8.7 cases/100,000 population [Table 2]) than during the 2002 epidemic, when attack rates were ≈100–250 cases per 100,000 population (*10*,*21*).

Table 2. Bacterial meningitis cases and incidence,Burkina Faso, 2007–2012*

| | Prevaccine | | | | Postvaccine | |
|---|---|---|---|---|---|---|
| Variable | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 |
| Total no. cases reported in MPE | NA | NA | NA | 3,413 | 3,415 | 5,807 |
| Total no. specimens tested in aggregated laboratory results | 550 | 93 | 241 | NA | NA | NA |
| Confirmed or probable cases, no. | 286 | 52 | 139† | 1,408 | 1,306 | 2,768 |
| *Neisseria meningitidis*, no. cases (Incidence): | 257 (1.8) | 49 (0.3) | 43 (0.3) | 170 (1.1) | 278 (1.7) | 1,788 (10.7) |
| *N. meningitidis* serogroup W, no. cases (Incidence): | 4 (<0.1) | 0 | 4 (<0.1) | 10 (<0.1) | 113 (0.7) | 1,451 (8.7) |
| Total cases reported in TLOH | 25,695 | 10,345 | 4,878 | 6,837 | 3,878 | 7,022 |

*Incidence = cases per 100,000 population. MPE, Maladies à Potentiel Epidémique (case-based surveillance); NA, not available; TLOH, Télégramme Lettre Official Hebdomadaire (aggregate case counts).
†Only latex agglutination results available.

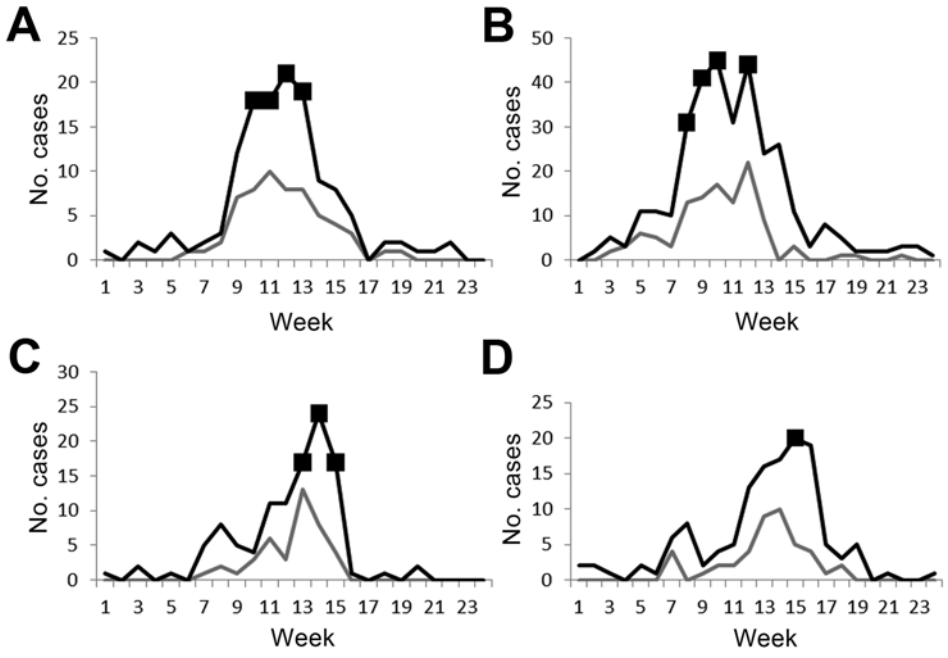


Figure 2. Total meningitis cases reported in Télégramme Lettre Official Hebdomadaire and laboratory-confirmed *Neisseria meningitidis* serogroup W cases for selected districts, Burkina Faso, 2012 meningitis season. A) District 1, population 150,000. B) District 2, population 300,000. C) District 3, population 100,000. D) District 4, population 200,000. Thin line indicates serogroup W cases; thick line, total cases; ▪ indicates weeks during which the number of cases crossed the weekly epidemic threshold. Data source: Télégramme Lettre Official Hebdomadaire and Maladies à Potentiel Epidémique.

The serogroup W clone associated with the Hajj-related outbreak during 2000 (*22*) has been present and a persistent cause of sporadic disease in Burkina Faso since the 2002 epidemic (R. Ouédraogo-Traoré, pers. comm.). The increase in serogroup W during 2012 might represent a natural increase in disease associated with waning population immunity in the 10 years since the last epidemic. This hypothesis is supported by the high proportion of serogroup W infections among younger children and the characterization of most serogroup W strains from 2012 as ST-11(cc11):P1.5,2:F1–1, the same genotype as the clone that caused the 2002 epidemic (*11*,*22*).

In Burkina Faso, rates of nasopharyngeal carriage of serogroup W are low (*23*). A previous study from Burkina Faso during the 2002 serogroup W epidemic demonstrated that serogroup W carriage infrequently induces protective immunity and that natural immunity against serogroup W may be lost (*24*). Serogroup replacement after nationwide vaccination with PsA-TT cannot be ruled out, however, and additional years of surveillance data, as well as nasopharyngeal carriage surveys, are required for a full understanding of whether serogroup replacement has resulted from PsA-TT vaccination. Preliminary data indicate that rates of serogroup W disease were lower during the 2013 meningitis season than during the 2012 meningitis season.

*N. meningitidis* has multiple genetic mechanisms to alter its antigenic profile, including capsular switching. Capsular switching has been observed among pneumococcal serotypes after the 7-valent pneumococcal conjugate vaccine was introduced in the United States, although the substantial declines in incidence of pneumococcal disease were maintained overall (*25*). Immediately after implementation of serogroup C meningococcal conjugate vaccine in the United Kingdom, no evidence of capsular replacement was found (*26*). Low rates of serogroup C disease in the United Kingdom have persisted, but serogroup Y has increased during the past several years in the United Kingdom and several other European countries (*27*). However, the effects of vaccination often are difficult to disentangle from natural fluctuations in meningococcal incidence and serogroup distribution.

With the recent shift in the epidemiology of bacterial meningitis in Burkina Faso after PsA-TT introduction, the current epidemic thresholds for predicting meningitis epidemics should be reevaluated. Although both serogroup W and serogroup X are known to cause epidemics, neither has been observed to cause the explosive epidemics of serogroup A or to have the periodicity observed for serogroup A epidemics. These thresholds were developed when the effects of disease were substantially higher so that epidemics could be anticipated and response activities (i.e., reactive vaccination campaigns) would prevent additional cases (*18*). In the 13 districts in Burkina Faso where meningitis reached the weekly epidemic threshold during 2012, the peak of reported cases was detected (Figure 2), but in no district was the cumulative annual epidemic threshold of 100 cases per 100,000 population met (*20*). During the 2012 meningitis season, 3 districts in Burkina Faso had vaccination campaigns that used quadrivalent polysaccharide vaccine. These are the first campaigns to use quadrivalent polysaccharide vaccine after PsA-TT vaccine; how many additional serogroup W cases were prevented and how administration of polysaccharide vaccination after PsA-TT will affect duration of protection against serogroup A disease are unknown.

The increases in serogroup W disease during 2012 were recognized because of the investment in high-quality meningitis surveillance in Burkina Faso, which underscores the importance of maintaining high-quality case-based meningitis surveillance capacity across the meningitis belt after PsA-TT introduction. Although additional years of data are needed to understand the epidemiology of meningococcal disease after PsA-TT introduction, it is evident that *N. meningitidis* will continue to cause sporadic disease and has potential to cause epidemics. In the meantime, countries need to effectively communicate the need for persons to remain diligent about seeking care for meningitis symptoms while not undermining public trust in the effectiveness of PsA-TT. The only available control strategy for serogroup W epidemics is reactive campaigns with trivalent or quadrivalent meningococcal polysaccharide vaccine. An affordable, multivalent vaccine, developed for use in the meningitis belt is needed in this disproportionately affected region to protect against epidemic meningitis.

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# Use of Drug-Susceptibility Testing for Management of Drug-Resistant Tuberculosis, Thailand, 2004–2008

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**Release date: February 19, 2014; Expiration date: February 19, 2015**

**Learning Objectives**

Upon completion of this activity, participants will be able to:

- Distinguish the clinical turnaround time for drug-susceptibility testing (DST) in the current study
- Evaluate physician actions based on the results of DST in the current study
- Analyze risk factors for inappropriate treatment of tuberculosis (TB) in the current study
- Analyze risk factors for worse clinical outcomes of TB treatment in the current study.

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In 2004, routine use of culture and drug-susceptibility testing (DST) was implemented for persons in 5 Thailand provinces with a diagnosis of tuberculosis (TB). To determine if DST results were being used to guide treatment, we conducted a retrospective chart review for patients with rifampin-resistant or multidrug-resistant (MDR) TB during 2004–2008. A total of 208 patients were identified. Median time from clinical sample collection to physician review of DST results was 114 days. Only 5.8% of patients with MDR TB were empirically prescribed an appropriate regimen; an additional 31.3% received an appropriate regimen after DST results were reviewed. Most patients with rifampin -resistant or MDR TB had successful treatment outcomes. Patients with HIV co-infection and patients who were unmarried or had received category II treatment before DST results were reviewed had less successful outcomes. Overall, review of available DST results was delayed, and results were rarely used to improve treatment.

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis*, is a global public health issue; 8.6 million incident cases and 1.3 million deaths were attributed to TB in 2012 (*1*). A severe threat to TB control is the emergence of multidrug-resistant (MDR) TB: worldwide, there are an estimated 650,000 MDR TB cases (*1*). To manage MDR TB, the World Health Organization (WHO) recommends empirically basing treatment on the general drug-susceptibility testing (DST) pattern of the population for patients in settings with limited laboratory capacity or for patients with pending DST results. Once laboratory results become available, WHO recommends treatment modification, as needed, according to the DST results (*2*).

Algorithms have been proposed to assist with clinical decision-making, but a proper laboratory diagnosis remains the benchmark for informing an effective and suitable treatment regimen (*3*). New technologies to more quickly detect TB, including drug-resistant TB, have become increasingly available (*4*–*8*). These tests are in use throughout the world, potentially improving the clinical management of TB by informing clinicians of which drugs may be most effective for individual patients. However, because of delays in receiving laboratory results (*3*), clinicians may have adopted a convention of patient clinical management that relies on epidemiologic data, medical history, and clinical signs and symptoms. Continuation of ineffective treatment causes excess patient illness and increases the potential for drug resistance, relapse, death, and transmission of drug-resistant *M. tuberculosis* strains. Earlier initiation of proper therapy may therefore result in substantial cost savings (*9*). Thus, with these new diagnostic technologies has come the critical need to examine how test results are being used in the clinical management of TB patients.

In 2004, the Thailand Ministry of Public Health–US Centers for Disease Control and Prevention Collaboration implemented routine liquid culture and DST of clinical samples for all persons with a diagnosis of TB disease in 5 provinces participating in the Active TB Surveillance Network (*10*). Clinicians were provided orientation to the new diagnostic tests and their interpretation and limitations. Although these procedures had been implemented, the extent to which clinicians were using DST results to inform treatment decision making was not known. During 2004–2008, we conducted a retrospective chart review to 1) determine sociodemographic, clinical, and laboratory characteristics of persons with a diagnosis of rifampin (RIF)-resistant TB or MDR TB (i.e., resistance to RIF and isoniazid [INH]); 2) determine the timing and use of DST results; 3) determine the effect of DST results on treatment regimens used for RIF-resistant and MDR TB; and 4) determine the association between treatment regimen characteristics and treatment outcomes.

## Materials and Methods

### Study Population

For the evaluation, we selected patients with DST results demonstrating infection with RIF-resistant or MDR TB who were registered for TB treatment during October 2004–September 2008 at health facilities within the Thailand TB Active Surveillance Network (*10*). The surveillance network included 7 health centers in the Bangkok Metropolitan Area and government hospitals in Chiang Rai, Phuket, Ubon Rachathani, and Tak Provinces. Patients from health facilities operated by private practitioners, nongovernmental organizations, or facilities serving solely as referral centers that do not manage ongoing treatment and outpatient care were excluded. Patients with incomplete laboratory data (e.g., missing date of specimen collection or missing DST results) and those with non-TB mycobacterium infection or a change in diagnosis were also excluded.

### Data Collection and Laboratory Testing

Trained clinic staff retrospectively collected patient information from routine medical and laboratory records onto standardized forms. For each patient, we recorded dates for the following: specimen collection for DST, receipt of DST results at the clinic, and first clinic visit for patients after DST results became available. We also recorded the date of each clinic visit, all drugs and dosages included in treatment regimens, and all treatment changes throughout the course of treatment.

Sputum specimens from patients were cultured at a provincial government laboratory by using Lowenstein-Jensen solid culture and Mycobacterial Growth Indicator Tube (MGIT) liquid culture (BACTEC 960, Becton-Dickinson, Franklin Lakes, NJ, USA) according to standard procedures (*11*). Isolates were sent to the Bangkok

or Thailand Ministry of Public Health National Reference Laboratory for identification and DST for first-line anti-TB drugs (i.e., streptomycin [STR], INH, RIF, and ethambutol [EMB]); Lowenstein-Jensen–based and MGIT-based methods were used for DST.

### Definitions and Treatment Regimens

Standard WHO definitions were used to categorize patients according to TB treatment history, site of TB infection, and treatment outcomes (*2*,*12*). Patients who completed treatment and those who were cured of TB were considered to have successful outcomes; patients for whom treatment failed and those who defaulted or died were considered to have poor treatment outcomes.

At the time this cohort of patients received a diagnosis and was clinically managed, national guidelines in Thailand recommended the use of standardized TB treatment regimens for MDR TB (*13*). These guidelines were not consistent with WHO guidelines (*2*); instead, they included only 3 months of a standard intensive-phase, injectable-based regimen and provided the option of using STR, rather than an aminoglycoside, as the injectable drug if the organism did not have documented STR resistance (*2*,*13*). Second-line drugs for MDR TB treatment were available to clinicians on request from a single source supported by the Department of Disease Control, Thailand Ministry of Public Health; the request process was not well standardized. For the purposes of this analysis, anti-TB treatment for MDR TB was considered appropriate if it was consistent with the Thailand national guidelines or if it was based on at least 3 drugs presumed to be effective according to the patient's first-line DST results (*13*). At the time, there were no specific recommendations for treatment of RIF-resistant TB in Thailand, and treating physicians were not required to follow a specific standard for drug-resistant TB treatment.

### Data Analysis

We used frequencies and summary statistics to describe patient characteristics, DST patterns, DST turnaround times, and treatment regimens prescribed for patients. Characteristics were assessed by each category of drug-resistance (RIF-resistant or MDR TB) and for the total sample population. Baseline characteristics of patients included in the analysis and of those excluded from analysis were compared by using the Wilcoxon-Mann-Whitney test for continuous variables and the $\chi^2$ test for categorical variables; these tests were also used to compare RIF-resistant and MDR TB patient groups in the analytic sample.

We used log-binomial analysis to calculate the odds ratios (ORs) and 95% CIs to evaluate the association between baseline demographic and clinical factors and prescription of an inappropriate treatment among patients with MDR TB. The analytic sample for evaluating the association between treatment characteristics and treatment outcomes was restricted to patients with final treatment outcomes available (excluding patients who transferred out or who were still receiving treatment) at the time of analysis. We also used log-binomial regression analysis to calculate the OR and 95% CI for the association between characteristics of the treatment regimen and final treatment outcomes for patients with RIF-resistant or MDR TB. All models were initially age-adjusted, and other factors were chosen for inclusion in multivariate analyses if the p value was <0.20 in bivariate analysis or if there was epidemiologic plausibility or previously published evidence suggesting an association with treatment outcomes. Collinearity and effect modification were assessed for all variables in the multivariate models. Significance was considered at $p \leq 0.05$. We used STATA version 10 (StataCorp, College Station, TX, USA) for all analyses.

## Results

### Patient and Clinical Characteristics

We identified a total of 490 patients as having TB that was RIF-resistant (n = 121) or MDR (n = 369) (Figure 1). Of these 490 patients, 208 (36 with RIF-resistant TB and 172 with MDR TB) were included in the evaluation. Patients with RIF-resistant TB who were excluded from analysis were substantially younger than those who were included in the analysis, and a substantially larger proportion of patients with HIV infection plus extrapulmonary or pulmonary and extrapulmonary disease were excluded from the RIF-resistant and MDR TB patient groups (Tables 1 and 2).

Among the 172 patients with MDR TB included in the analysis, 89 (51.7%) were new TB patients, 60 (34.9%) were retreatment patients, and 10 (5.8%) transferred in from another facility (Table 2). Among the 36 patients with RIF-resistant TB, 26 (72.2%) had newly diagnosed infection and 9 (25.0%) were retreatment patients (Table 2). The median age of patients with RIF-resistant and MDR TB was 42 years (interquartile range [IQR] 34–58) and 39 years (IQR 29–50), respectively (Table 1). Overall, baseline characteristics were comparable for patients with RIF-resistant and MDR TB. Most patients in both groups were male, married, and HIVseronegative.

### Drug-Susceptibility Patterns

Of the 36 patients with RIF-resistant TB, 28 (77.8%) had resistance to RIF alone. Another 4 (11.1%) also had resistance to RIF and STR; 2 (5.6%) had resistance to RIF and EMB; and 2 (5.6%) had resistance to RIF, EMB, and STR.

Among the 172 patients with MDR TB, 69 (40.1%) had resistance to only INH and RIF. Another 13 (7.6%) had

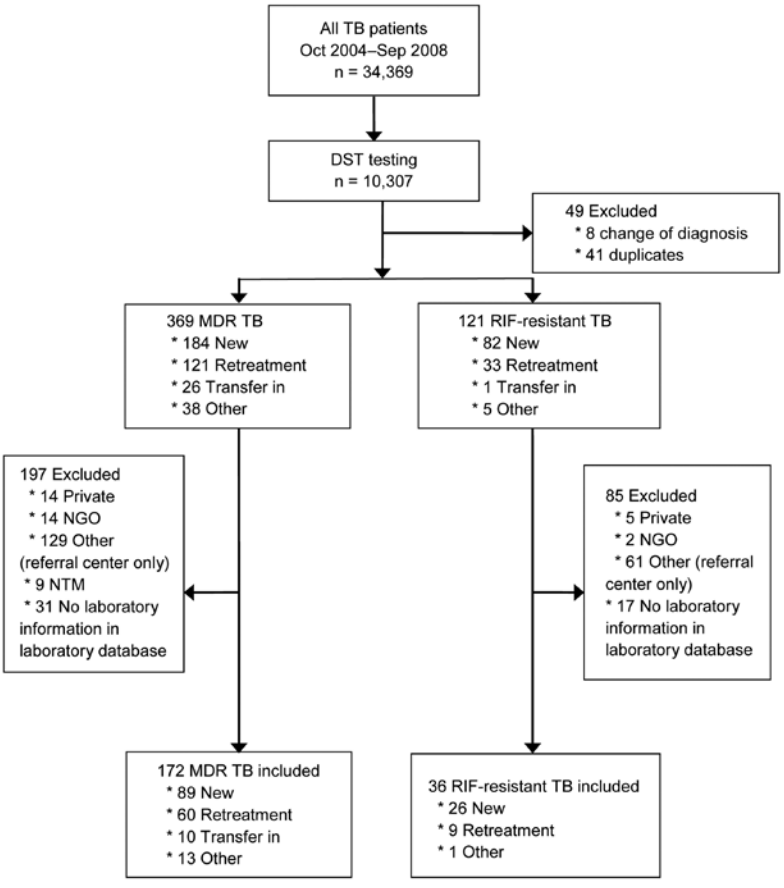


Figure 1. Selection of patients for an analysis of drug-susceptibility testing and management of drug-resistant tuberculosis, Thailand, October 2004–September 2008. Patients had rifampin- or multidrug-resistant tuberculosis and were from 5 Thailand provinces participating in the Thailand Active TB Surveillance Network. TB, tuberculosis; DST, drug-susceptibility testing; MDR, multidrug resistant; RIF, rifampin; Private, patient from private hospital; NGO, patient from nongovernmental hospital; NTM, nontuberculosis mycobacterium.

resistance to INH, RIF, and EMB; 55 (32.0%) had resistance to INH, RIF, and STR; and 35 (20.3%) had resistance to STR and EMB.

### Turnaround for Drug-Susceptibility Testing Results

The median time from collection of patient sputum samples to clinic receipt of DST results was 97.5 days (IQR 66–133.3) (Table 3). The median time from clinic receipt of DST results to physician review of results at the first post-DST visit was 7 days (IQR 1–21). Overall, the median time from sputum collection to the first physician review of the DST result was 109.5 days (IQR 73–150). Patients with MDR TB had longer diagnostic turnaround times than patients with RIF-resistant TB.

### TB Treatment

Of the 172 patients with MDR TB included in the analysis, 10 (5.8%) were initially prescribed an appropriate treatment regimen, and 51 (29.7%) were prescribed appropriate treatment at some point during the treatment course. Forty-one patients with MDR TB and 13 with RIF-resistant TB were not eligible for treatment changes after clinic results became available: 31 of these MDR TB and all 13 patients with RIF-resistant TB had treatment outcomes before the return of DST results, and the other 10 patients with MDR TB were empirically treated with appropriate drugs.

Of the remaining 131 patients with MDR TB, 37 (28.2%) never had a treatment change, 53 (40.5%) had a treatment change at the first clinic visit following availability of DST results, and 41 (31.3%) had changes made later in the treatment course. Of the 53 patients with a change made at the first post-DST visit, 24 (45.3%; 18.3% of those eligible for change) were prescribed on appropriate regimens; 9 of the 24 changes were in accord with the national treatment guidelines, and the 15 other changes were to ≥3 drugs presumed to be effective (Figure 2). Of the remaining 29 MDR TB patients who had a regimen change at the first post-DST visit, 3 were changed to a category I regimen and 26 were placed on a nonstandard second-line treatment combination. By 3 months after the first physician review of the DST results, 51.7% of these 131 patients with MDR TB received changes to their treatment plan, of which ≈20% were appropriate changes. At 12 months and onward after the first physician review of DST results, ≈85% of patients with MDR TB had changes to their treatment, of which ≈30% were classified as appropriate adjustments.

Of the 23 patients with RIF-resistant TB who were eligible for treatment changes, 4 (17.4%) had a treatment

Table 1. Baseline demographic characteristics for patients with drug-resistant TB, Thailand, 2004–2008*

| Characteristic | Patients with RIF-resistant TB, n = 121 | | | Patients with MDR TB, n = 369 | | | Patients with RIF-resistant vs. MDR TB, p value‡ |
|---|---|---|---|---|---|---|---|
| | Included, n = 36 | Excluded, n = 85 | p value† | Included, n = 172 | Excluded, n = 197 | p value† | |
| Age, median (IQR), y | 42 (34–58) | 36 (29–48) | 0.02 | 39 (29–50) | 36 (28–47) | 0.11 | 0.10 |
| Sex | | | | | | | |
| M | 22 (61.1) | 55 (64.7) | 0.71 | 117 (68.0) | 128 (65.0) | 0.58 | 0.42 |
| F | 14 (38.9) | 30 (35.3) | | 55 (32.0) | 68 (34.5) | | |
| Data missing | 0 (0.0) | 0 (0.0) | | 0 (0.0) | 1 (0.5) | | |
| Marital status | | | | | | | |
| Married | 22 (61.1) | 39 (46.4) | 0.32 | 93 (54.1) | 85 (43.1) | 0.001 | 0.06 |
| Single/divorced/widowed | 13 (36.1) | 43 (51.2) | | 79 (45.9) | 98 (50.0) | | |
| Data missing | 1 (2.8) | 2 (2.4) | | 0 (0.0) | 13 (6.6) | | |
| Nationality | | | | | | | |
| Thai | 33 (91.7) | 76 (89.4) | 0.70 | 144 (83.7) | 170 (86.3) | 0.49 | 0.22 |
| Not Thai | 3 (8.3) | 9 (10.6) | | 28 (16.3) | 27 (13.7) | | |

*Patients were from the Thailand TB Active Surveillance Network and were either included or not included in the current study. Data are no. (%) unless otherwise indicated. TB, tuberculosis; RIF, rifampin; MDR, multidrug-resistance; IQR, interquartile range.
†p values reflect comparison between included and excluded patients in each TB drug resistance group.
‡p values reflect comparison between included RIF-resistant patients and MDR TB patients.

change at the first post-DST visit, and the remaining 19 (82.6%) patients had changes made during subsequent post-DST clinic visits. Of the 4 patients with RIF-resistant TB who received regimen changes at the first post-DST visit, 2 were changed to a nonstandard first-line treatment combination (INH + pyrazinamide + EMB + STR or INH + EMB), 1 was changed to a nonstandard second-line treatment combination (INH + EMB + ofloxacin), and 1 discontinued treatment due to hepatic cirrhosis. Figure 2 is restricted to MDR TB patients because there was no written guideline on appropriate treatment of patients with RIF-resistant TB in Thailand during the study period; therefore, we were unable to differentiate between appropriate and inappropriate changes for the patients with RIF-resistant TB.

### Baseline Factors and Appropriateness of Treatment

Examination of baseline factors associated with prescription of an inappropriate treatment for MDR TB case-patients indicated that retreatment patients were significantly more likely than new patients to be prescribed an inappropriate regimen (age-adjusted OR 2.6, 95% CI 1.0–6.3; p = 0.04) (Table 4). A significant association was not identified between the time delay between sputum collection and the first clinic visit following availability of DST results and whether patients were prescribed appropriate regimens.

### Patient Group and Treatment Outcome

Twelve patients (2 with RIF-resistant TB, 10 with MDR TB) with treatment outcomes that indicated they had transferred out and 2 patients with MDR TB who were still receiving treatment at the time of the evaluation were excluded from the final analytic sample (final n = 194; 34 RIF-resistant TB and 160 MDR TB cases). Treatment success was slightly greater among the RIF-resistant TB group than the MDR TB group (76.5% vs. 60.6%, p = 0.08). Patients who were not married (adjusted OR 2.3, 95% CI 1.2–4.6; p = 0.01), who were HIV positive (adjusted OR 2.2, 95% CI 1.1–4.4; p = 0.04), and who received category II treatment before receiving DST results (adjusted OR 2.6, 95% CI 1.1–6.4; p = 0.05) had poorer treatment outcomes (Table 5, Appendix, wwwnc.cdc.gov/EID/article/20/3/13-0951-T5.htm). In the analysis restricted to patients with MDR TB, receiving inappropriate treatment was not significantly associated with poor treatment outcomes (OR = 0.77, 95% CI 0.3–1.8; p = 0.55).

## Discussion

This evaluation revealed that most treatment regimens assigned to patients with RIF-resistant or MDR TB in selected areas of Thailand were not based on DST results. Less than one third of patients with MDR TB received appropriate treatment, and patients who had previously received treatment for TB were >2 times more likely to be prescribed an inappropriate treatment regimen. When DST results were available, treatment changes did not necessarily reflect nationally recommended standard regimens for drug-resistant TB or the resistance profile of the infecting TB strain. In some cases, physicians probably did not change to second-line treatment because of the clinical condition of the patient; only 16% of patients overall had smear-positive test results at month 5 (data not shown). Persistence of smear-negative test results among identified MDR TB cases has been cited as a reason for not changing to a standardized MDR TB treatment regimen; other reasons have included patient loss to follow-up, patient death, and patient refusal to change treatment (*14*). A study evaluating the influence of the microscopic observation drug susceptibility (MODS) assay, which allows for determination of drug susceptibility directly from sputum in just 7–10 days, on the clinical management of TB patients also reported that even when indicated, appropriate treatment regimen changes were not always made (*15*). MDR TB treatment is

Table 2. Baseline clinical characteristics for patients with drug-resistant TB, Thailand, 2004–2008*

| Characteristic | No. (%) patients with RIF-resistant TB, n = 121 | | | No. (%) patients with MDR TB, n = 369 | | | Patients with RIF-resistant vs. MDR TB, p value‡ |
|---|---|---|---|---|---|---|---|
| | Included, n = 36 | Excluded, n = 85 | p value† | Included, n = 172 | Excluded, n = 197 | p value† | |
| Case status | | | | | | | |
| New | 26 (72.2) | 56 (65.9) | 0.64 | 89 (51.7) | 95 (48.2) | 0.51 | 0.17 |
| Retreatment after relapse | 6 (16.7) | 19 (22.4) | | 25 (14.5) | 24 (12.2) | | |
| Retreatment after failure | 2 (5.6) | 1 (1.2) | | 21 (12.2) | 19 (9.6) | | |
| Retreatment after default | 1 (2.8) | 4 (4.7) | | 14 (8.1) | 18 (9.1) | | |
| Transfer in | 0 (0.0) | 1 (1.2) | | 10 (5.8) | 16 (8.1) | | |
| Other | 1 (2.8) | 4 (4.7) | | 13 (7.6) | 25 (12.7) | | |
| Site of TB | | | | | | | |
| Pulmonary | 33 (91.7) | 61 (71.8) | 0.05 | 160 (93.0) | 162 (82.2) | 0.008 | 0.69 |
| Extrapulmonary | 2 (5.6) | 13 (15.3) | | 5 (2.9) | 15 (7.6) | | |
| Both | 1 (2.8) | 11 (12.9) | | 7 (4.1) | 20 (10.2) | | |
| Cough ≥2 wk | | | | | | | |
| No | 11 (30.6) | 39 (45.9) | 0.22 | 40 (23.3) | 61 (31.0) | <0.001 | 0.36 |
| Yes | 25 (69.4) | 45 (52.9) | | 132 (76.7) | 123 (62.4) | | |
| Data missing | 0 (0.0) | 1 (1.2) | | 0 (0.0) | 13 (6.6) | | |
| Smear status | | | | | | | |
| Negative | 4 (11.1) | 11 (12.9) | 0.44 | 26 (15.1) | 26 (13.2) | 0.57 | 0.71 |
| Positive | 30 (83.3) | 63 (74.1) | | 140 (81.4) | 160 (81.2) | | |
| Data missing | 2 (5.6) | 11 (12.9) | | 6 (3.5) | 11 (5.6) | | |
| Chest radiograph | | | | | | | |
| Normal | 0 (0.0) | 4 (4.7) | 0.27 | 4 (2.3) | 10 (5.1) | 0.17 | 0.79 |
| Abnormal, no cavity | 22 (61.1) | 41 (48.2) | | 109 (63.4) | 104 (52.8) | | |
| Abnormal, with cavity | 8 (22.2) | 29 (34.1) | | 34 (19.8) | 48 (24.4) | | |
| Data missing | 6 (16.7) | 11 (12.9) | | 25 (14.5) | 35 (17.8) | | |
| HIV status | | | | | | | |
| Negative | 23 (63.9) | 29 (34.1) | 0.004 | 112 (65.1) | 92 (46.7) | 0.001 | 0.17 |
| Positive | 13 (36.1) | 46 (54.1) | | 47 (27.3) | 75 (38.1) | | |
| Data missing | 0 (0.0) | 10 (11.8) | | 13 (7.6) | 30 (15.2) | | |
| Outcome | | | | | | | |
| Treatment success | | | | | | | |
| TB cured | 20 (55.6) | 26 (30.6) | 0.25 | 51 (29.7) | 33 (16.8) | 0.06 | 0.07 |
| Treatment completed | 5 (13.9) | 21 (24.7) | | 25 (14.5) | 36 (18.3) | | |
| Poor outcome | | | | | | | |
| Treatment failed | 1 (2.8) | 5 (5.9) | | 31 (18.0) | 29 (14.7) | | |
| Patient defaulted | 3 (8.3) | 10 (11.8) | | 17 (9.9) | 28 (14.2) | | |
| Patient died | 5 (13.9) | 12 (14.1) | | 30 (17.4) | 40 (20.3) | | |
| Transferred out | 2 (5.6) | 9 (10.6) | | 15 (8.7) | 23 (11.7) | | |
| Ongoing treatment | 0 | 2 (2.4) | | 3 (1.7) | 8 (4.1) | | |

*Patients were from the Thailand TB Active Surveillance Network and were either included or not included in the current study. TB, tuberculosis; RIF, rifampin; MDR, multidrug-resistance.
†p values reflect comparison between included and excluded patients in each TB drug resistance group.
‡p values reflect comparison between included RIF-resistant and MDR TB patients.

highly decentralized in Thailand, and some clinicians may not have been familiar with treatment guidelines.

The median delay from the time of sputum collection to the time DST results were available at the clinic exceeded 14 weeks, and further delays were noted between availability of results and a clinical encounter. During the evaluation period, MGIT liquid culture was used for diagnosis of TB and of drug resistance; the turnaround time for culture results is generally 4–6 weeks (*4*). Other studies have also demonstrated the effect of clinic delays on TB management, even when laboratory results are available in a timely manner (*9*,*14*,*16*). The time interval for diagnosing RIF-resistant and MDR TB in this evaluation was longer than expected, probably due to constraints with specimen transport, laboratory capacity, and administrative delays in providing results to clinics. In addition, MDR TB result reporting was further delayed because the implications for regimen change were considered more serious for MDR TB than for RIF-resistant TB; the reference laboratories tended to hold MGIT-based DST results until they were confirmed by solid culture. Our findings highlight these other sources of delay beyond those intrinsic to a given assay as pivotal for ensuring the benefits of rapid diagnostic technologies.

In this evaluation, patients with MDR TB were more likely to receive an inappropriate initial treatment regimen if they were a retreatment patient rather than a new patient. This finding suggests that retreatment cases should be prioritized when considering the application of rapid diagnostic technologies, and actions should be taken to expedite the transport and testing of specimens and reporting results to the clinician. In addition, patients who initially received category II treatment were significantly more

Table 3. Diagnostic turnaround for DST results for 130 RIF-resistant and MDR TB patients, Thailand 2004–2008*

| Turnaround variable | Median (IQR), d | | |
|---|---|---|---|
| | RIF-resistant TB (n = 18) | MDR TB (n = 112) | Total RIF-resistant and MDR TB |
| Time from sputum collection to clinic receipt of results | 75.0 (49.0–112.0) | 100.0 (67.3–137.5) | 97.5 (66.0–133.3) |
| Time from clinic receipt of results to review by physician† | 9.0 (0.8–16.5) | 7.0 (1.0–21.8) | 7.0 (1.0–21.0) |
| Time from sputum collection to result review by physician† | 83.0 (53.0–130.3) | 111.0 (77.3–153.3) | 109.5 (73.0–150.0) |

*The 130 TB patients represented here were among 208 patients from 5 Thailand provinces participating in the Thailand Active TB Surveillance Network. Calculations were restricted to patients who had complete information for sputum collection date, date of receipt of DST at the clinic, and date of the first physician visit after availability of DST results. Three MDR TB patients were missing initial sputum collection date, 14 RIF-resistant and 41 MDR TB patients were missing date of receipt of DST results at the clinic, and 16 RIF-resistant and 47 MDR TB patients were missing date of first clinic visit following receipt of DST results. DST, drug susceptibility test; RIF, rifampin; MDR, multidrug-resistant; TB, tuberculosis; IQR, interquartile range.
†Represents first post-DST clinic visit.

likely to default, fail treatment, or die. This finding is consistent with those of previous studies demonstrating the association between category II treatment and poor outcomes and the growing body of evidence advocating for the elimination of the category II retreatment regimen (*17–21*).

Multiple studies have reported high rates of treatment success among patients prescribed individualized regimens tailored to DST results (*22–27*). In a recent meta-analysis of 33 studies in 20 countries evaluating MDR TB treatment outcomes, individualized treatment had higher treatment success compared with standardized regimens based on local drug-susceptibility patterns (64 vs. 54%), although the difference was not statistically significant (*28*). In our study, the lack of direct association between the appropriateness of the treatment regimen and treatment outcomes among patients with MDR TB may have been due to the small number of patients prescribed appropriate regimens during the treatment course, or it may be that treatment decisions based on other clinical factors were more pertinent to determining outcomes.

Our analysis has limitations. First, patients were excluded if they had incomplete laboratory or clinic data, including patients for whom the date of specimen collection or receiving or reviewing the DST results at the clinic were not recorded. However, we do not have any indication that the omission of this information was systematic. Second, it is possible that our conclusions are not representative of all patients in Thailand with RIF-resistant or MDR TB. We noted that a higher proportion of patients in the excluded group than in the analytic sample were HIV positive and had extrapulmonary TB; this disproportion possibly occurred because of our inclusion requirement of complete laboratory data, and the microbiological yield from these 2 clinical groups is often low. In addition, one referral facility with a high proportion of HIV-associated TB cases was excluded because as a facility providing episodic tertiary consultation, they rarely have complete diagnostic and treatment data for patients. Last, the data for our study were abstracted from medical charts and routine surveillance not intended for specific research purposes; it is possible that some drug adjustments were not identified. Because of the retrospective study design, detailed information on factors considered in clinical decision-making and treatment prescriptions for patients was not available if it was not explicitly documented in the medical records. DST is only one component considered in prescribing treatment; the patient's clinical status and risks involved with alternate drugs are also key factors. The long delays in obtaining DST findings may result in a heavier reliance on clinical factors for prescribing decisions.

Future research that identifies reasons for the low utilization of laboratory results when prescribing anti-TB therapy will help to develop interventions that can facilitate optimal treatment for drug-resistant TB. Furthermore, evaluation is needed to determine where and why delays

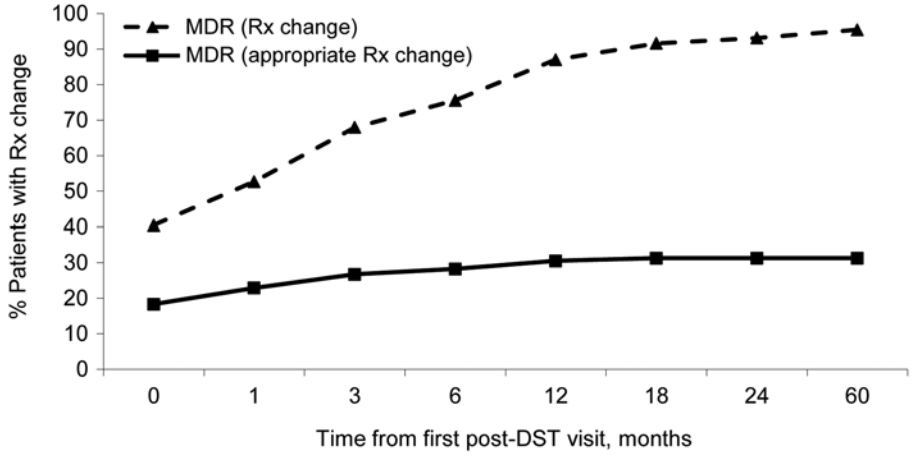


Figure 2. Percentage of MDR-TB patients who were eligible for a treatment regimen change (n = 131) who received a change, according to time from the first review of DST result by the physician, TB Active Surveillance Network, Thailand 2004–2008. Rx, prescription treatment. DST, drug-susceptibility testing; MDR, multidrug-resistant TB.

Table 4. Association between baseline sociodemographic and clinical characteristics and prescription of an inappropriate MDR TB treatment regimen for 172 MDR TB patients 2004–2008*

| Characteristic | Univariate OR (95% CI) | p value | Multivariate OR (95% CI)† | p value |
|---|---|---|---|---|
| Age, y | | | | |
| <45 | Reference | | | |
| ≥45 | 0.54 (0.2–1.2) | 0.13 | | |
| Sex | | | | |
| M | Reference | | | |
| F | 0.74 (0.3–1.8) | 0.49 | | |
| Marital Status | | | | |
| Married | Reference | | | |
| Single/divorced/widowed | 0.44 (0.2–1.0) | 0.06 | | |
| Nationality | | | | |
| Thai | Reference | | | |
| Non-Thai | 1.0 (0.4–3.0) | 0.95 | | |
| Case status | | | | |
| New | Reference | | Reference | |
| Retreatment | 2.4 (1.0–5.9) | 0.05 | 2.6 (1.0–6.3) | 0.04 |
| Transfer in/other | 2.8 (0.9–8.7) | 0.08 | 2.9 (0.9–9.3) | 0.07 |
| Site of TB | | | | |
| Pulmonary | Reference | | | |
| Extrapulmonary or both | 0.41 (0.1–3.3) | 0.40 | | |
| Cough ≥2 wk | | | | |
| No | Reference | | | |
| Yes | 1.6 (0.6–4.6) | 0.35 | | |
| Smear status | | | | |
| Negative | Reference | | | |
| Positive | 6.5 (0.8–50.2) | 0.07 | | |
| Cavitation seen on chest x-ray | | | | |
| No | Reference | | | |
| Yes | 0.40 (0.1–1.4) | 0.16 | | |
| HIV status | | | | |
| Negative | Reference | | | |
| Positive | 0.38 (0.1–1.2) | 0.09 | | |
| Resistance pattern | | | | |
| INH + RIF | Reference | | | |
| INH + RIF + EMB or INH + RIF + STR | 0.80 (0.3–2.1) | 0.65 | | |
| INH + RIF + EMB + STR | 2.1 (0.8–5.6) | 0.13 | | |
| Delay from sputum collection to firs post-DST visit, d | | | | |
| 35–74 | Reference | | | |
| 75–112 | 1.3 (04–4.6) | 0.70 | | |
| 113–155 | 1.8 (0.5–6.2) | 0.38 | | |
| >155 | 1.0 (0.3–3.8) | 0.97 | | |

*The patients were from 5 Thailand provinces participating in the Thailand Active TB Surveillance Network. OR, odds ratio; MDR, multidrug resistant; TB, tuberculosis; INH, isoniazid; RIF, rifampin; EMB, ethambutol; STR, streptomycin; DST, drug-susceptibility testing.
†Adjusted for age as a continuous variable.

unrelated to assay result turnaround times occurred; such delays may occur during specimen transport or processing, or they may be related to the timing of clinic notifications or the review of results by clinic physicians. Physicians' knowledge of the national guidelines and treatment algorithms as well as their ability to interpret and use DST results to improve treatment regimens should be assessed. Assessment of DST result uptake in other health sectors (e.g., private practice, nongovernmental organizations, and referral centers) would be informative because several participants who were at high risk for treatment failure (i.e., patients with HIV infection or extrapulmonary disease) were excluded from the current study.

In conclusion, utilization of DST results in the clinical management of patients with RIF-resistant or MDR TB was poor in Thailand during 2004–2008. Since the time of this evaluation, access to second-line drugs has improved in Thailand: the request process has been streamlined and standardized, and the national treatment guidelines have been clarified and strengthened and disseminated to clinicians. Attention to the DST reporting system has also reduced delays somewhat. These factors will need to be considered in assessing the effect of more rapid molecular testing methods.

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# Comparison of Imported *Plasmodium ovale curtisi* and *P. ovale wallikeri* Infections among Patients in Spain, 2005–2011

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Sequencing data from *Plasmodium ovale* genotypes co-circulating in multiple countries support the hypothesis that *P. ovale curtisi* and *P. ovale wallikeri* are 2 separate species. We conducted a multicenter, retrospective, comparative study in Spain of 21 patients who had imported *P. ovale curtisi* infections and 14 who had imported *P. ovale wallikeri* infections confirmed by PCR and gene sequencing during June 2005–December 2011. The only significant finding was more severe thrombocytopenia among patients with *P. ovale wallikeri* infection than among those with *P. ovale curtisi* infection (p = 0.031). However, we also found nonsignificant trends showing that patients with *P. ovale wallikeri* infection had shorter time from arrival in Spain to onset of symptoms, lower level of albumin, higher median maximum core temperature, and more markers of hemolysis than did those with *P. ovale curtisi* infection. Larger, prospective studies are needed to confirm these findings.

Malaria caused by *Plasmodium ovale* infection has been considered a low-prevalence disease with limited geographic distribution, benign clinical course, and easy treatment; therefore, little attention has been paid to it. Diagnosis of *P. ovale* malaria can be difficult because of low parasitemia levels, mixed infections with other *Plasmodium* species, and false negatives from malaria rapid diagnostic tests (RDTs) (*1*). However, recent epidemiologic studies conducted by using PCR techniques have found *P. ovale* infections in most of sub-Saharan Africa, Southeast Asia, and the Indian subcontinent (*2*–*5*), including prevalence as high as 15% according to results of cross-sectional studies conducted in rural Nigeria (*6*) and Papua New Guinea (*7*). In addition, severe complications such as spleen rupture, severe anemia, or acute respiratory distress syndrome (ADRS) (*8*) may occur in patients with *P. ovale* malaria. Thus, the global burden of *P. ovale* infection might have been underestimated.

On the basis of differences in its gene sequences, *P. ovale* was considered to be dimorphic or to comprise 2 subspecies (*2*,*3*,*9*,*10*). This difference has hampered molecular diagnosis in some cases because of lack of DNA amplification by PCR with gene-specific primers for the small subunit ribosomal RNA (ssrRNA) (*10*). These subspecies had been named classic and variant *P. ovale*, but a comprehensive study recently described differences between these subspecies in at least 6 genes (*4*). These findings demonstrate that *P. ovale* actually consists of 2 subspecies that cocirculate in Africa and Asia and that are unable to recombine genetically; the differences seem to be explained by real biological factors, rather than ecologic or geographic factors (*11*). *P. ovale curtisi* and *P. ovale wallikeri* were the names proposed for these species (*4*).

Scant information is available on differences in clinical and analytical features, relapse profile, or accuracy of RDT results between these proposed species. Relatively high parasitemia levels were found in some patients with *P. ovale wallikeri* infection in Thailand (*12*), Vietnam (*13*), and Flores Island (*14*). A study published from a disease-endemic area of Bangladesh reported on the clinical features and degree of parasitemia in 13 patients with *P. ovale wallikeri*

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infection and 10 with *P. ovale curtisi* infection (*5*). These infections were diagnosed by PCR; only 4 of the 23 patients were symptomatic. Another recent study compared parasitemia levels, RDT results, and patient country of origin for 31 patients from Côte d'Ivoire and the Comoros Islands with imported *P. ovale wallikeri* infection and 59 with *P. ovale curtisi* infection, but no clinical data were provided (*15*). Clearly, information on these infections is limited.

Growth in international travel and migration has increased the incidence of imported malaria in industrialized countries. *P. ovale* infection may represent up to 8% of imported malaria cases, according to some published series of patients primarily from West Africa (*16*,*17*), where the proportion of sub-Saharan immigrants is high and PCR has been systematically performed. Yet, it is difficult to gather a substantial number of cases with clinico-epidemiologic correlation and molecular data. To identify clinical or analytical differences between *P. ovale wallikeri* and *P. ovale curtisi* infections and expand data on these infections, we conducted a multicenter, retrospective, comparative study of imported *P. ovale* infections diagnosed in Spain during 2005–2011.

## Methods

### Sample Selection

During June 2005–December 2011, blood samples from all patients with positive PCR results for imported infection with *P. ovale* were sent from public hospitals in Spain to the reference Malaria & Emerging Parasitic Diseases Laboratory of the National Centre of Microbiology in Madrid. The samples were shipped to the laboratory 1) to confirm the diagnosis of malaria and the species or 2) to study fever, anemia, or suspected malaria in patients with negative results on thick and/or thin smears and RDTs who were considered at high risk for malaria (i.e., immigrants and travelers to malaria-endemic areas).

### Microbiological Diagnosis

The initial diagnosis of imported *Plasmodium* spp. infection was made by thick and/or thin smears and/or by using the second-generation RDT NOW Malaria Test Kit (Binax Inc., Scarborough, ME, USA) for histidine-rich protein 2 antigen of *P. falciparum* and aldolase enzyme common to all *Plasmodium* spp. Blood smears were stained by a standard technique with Giemsa solution for 30 min and were reviewed by an expert microbiologist. Parasite count was measured by determining the proportion of parasitized erythrocytes or the number of trophozoites per microliter.

### Isolation of Parasite DNA and Molecular Diagnosis Confirmation

DNA isolation from whole blood was performed by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. *P. ovale* molecular diagnosis was confirmed by using seminested multiplex malaria PCR (*18*), which enables the discernment of the 4 most prevalent human malaria species by amplified fragments of DNA in 2 sequential PCRs.

### *P. ovale* Subtype Characterization and Confirmation

Partial sequencing of the ssrRNA gene was used to differentiate *P. ovale curtisi* from *P. ovale wallikeri*. ssrRNA amplification was performed by using a nested PCR specific for *Plasmodium*. The first reaction included UNR (5′-GACGGT ATCTGATCGTCTTC-3′) and PLF (5′-AGTGTGTATCCAATCGAGTTTC-3′) primers, which correspond to the first reaction of the seminested multiplex malaria PCR. The second reaction incorporated the products of the first reaction, along with NewPLFsh (5′-CTATCAGCTTTTGATGTTAG-3′) and NewRevsh (5′-CCTTAACTTTCGTTCTTG-3′) primers. Infection with different malaria species yielded products of 710–740 bp.

The PCR mixture in both reactions consisted of 75 mmol/L Tris-HCl (pH 9.0), 2 mmol/L $MgCl_2$, 50 mmol/L KCl, 20 mmol/L $(NH_4)_2SO_4$, 200 μmol/L dNTP, 0.075 μmol/L of the corresponding PCR primers, 1.25 units *Taq* DNA polymerase (Biotools B&M Labs, S.A., Madrid, Spain), and the template DNA in a reaction volume of 50 μL. The amount of template was 5 μL of DNA extracted by using a QIAamp DNA Blood Mini Kit (QIAGEN). For the second reaction mixture, 2 μL of the PCR product of the first reaction was used as template. For both reactions, a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used, beginning with 7 min at 94°C, followed by 40 cycles of 20 s at 94°C, 20 s at 62°C, and 30 s at 72°C for the first round; or 35 cycles of 20 s at 94°C, 20 s at 53°C, and 20 s at 72°C for the second round. The final cycle was followed by an extension time of 10 min at 72°C.

The amplified products were purified by using Illustra DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and sequenced by using the Big Dye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3700 DNA Analyzer (Applied Biosystems). All amplified products were sequenced in both directions twice. To confirm *P. ovale* subtyping, a nested PCR amplification plus sequencing targeting cytochrome (Cyt) b was performed (*3*) in 3 samples of each group, by using a unique second amplification (nested) reaction with primers Cyt b 2F and Cyt b 2R.

### Data Collection

Hospitals that submitted PCR-confirmed and *P. ovale* subtype–identified samples were invited to collaborate in the study. A database was designed and completed after the retrospective review of medical reports and laboratory

registries. Patient data collected included sex, age, ethnicity, underlying diseases, time living in non–malaria-endemic countries, dates and purpose of travel, countries visited, malaria chemoprophylaxis, date of admission and diagnosis, symptoms and clinical signs, physical examinations, and complications of severe malaria according to World Health Organization criteria (*19*). The closest possible date of infection was defined as the day of departure from a malaria-endemic area. The time between date of arrival in Spain and onset of illness or diagnosis was calculated once asymptomatic patients were excluded. Patients were classified as early immigrant if they had stayed in a country without malaria for <1 year before diagnosis and as visiting friends and relatives if they had traveled to a malaria-endemic country after 1 year living in a non–malaria-endemic area. Recent *Plasmodium* infection was defined as probable or definite malaria infection in the 3 months before *P. ovale* infection was diagnosed.

Recorded laboratory results included microbiological data; complete blood count with leukocytes, hemoglobin, and platelet levels; levels of creatinine, albumin, transaminases, lactate dehydrogenase, and direct bilirubin in plasma; and glucose-6-phosphate dehydrogenase activity in erythrocytes. Abdominal ultrasonography and serologic studies to detect infection with HIV and hepatitis A, B, and C viruses were reviewed. Recorded treatments and compliance, clinical and microbiological evolution, and duration of hospital stay for those admitted were included.

### Statistical Analysis

Differences of proportions were evaluated by $\chi^2$ test or Fisher exact test, as appropriate for sample size. Means between groups were calculated by using the Student *t*-test for independent samples if the normal distribution could be assumed; we used the Levene test for homogeneity of variances. If normality was not valid, we used the Mann-Whitney U test for nonparametric variables.

To test for normality, we used either the Shapiro-Wilks test for small samples or the Kolmogorov-Smirnov test with the Lilliefors correction for large samples. Values were reported as means and SDs or, for nonparametric distributions, medians and interquartile ranges (IQRs). A 2-sided p value of <0.05 was considered to indicate statistical significance. Statistical analyses were performed by using SPSS version 15 (SPSS Inc., Chicago, IL, USA).

## Results

During June 2005–December 2011, a total of 102 samples positive by PCR for *P. ovale* were analyzed at the reference laboratory; of these, we were able to amplify and genotype 55 samples. Poor quality of long-term stored DNA prevented amplification of the other samples. Genetic analyses of the *cytb* gene identified *P. ovale curtisi* in 31 samples from 28 patients and *P. ovale wallikeri* in 24 samples from to 22 patients.

Twelve hospitals agreed to participate in the study and provided complete epidemiologic, microbiological, biochemical, clinical, and therapeutic information for 35 of the 50 patients for which *P. ovale* genetic characterization was available. Of these, 21 patients had *P. ovale curtisi* infection and 14 had *P. ovale wallikeri* infection. Table 1 shows the demographic and epidemiologic data for these 35 patients. Patient age and sex were virtually the same for patients with *P. ovale curtisi* and *P. ovale wallikeri* infections and corresponded mostly to young persons from Africa who traveled to visit their friends and relatives for a long period or immigrants who had recently arrived.

The lapses between time of arrival in Spain, onset of illness, and diagnosis were longer for patients with *P. ovale curtisi* infection than for those with *P. ovale wallikeri* infection, but not significantly. Most travelers did not take any malaria prophylaxis or did not adhere to the full regimen. All but 2 infections were acquired in West Africa, and both *Plasmodium s*pecies were found in patients from Nigeria, Equatorial Guinea, Ghana, and Guinea-Conakry. A high rate of underlying disease was found among patients in both parasite groups; the *P. ovale curtisi* group included 3 early immigrants who were chronically infected with HIV, hepatitis B virus, and hepatitis C virus and carried filarial and intestinal parasites.

Microbiological and laboratory data for the patients are shown in Table 2. Statistically significant worse levels of thrombocytopenia were found among patients with *P. ovale wallikeri* infection compared with those who had *P. ovale curtisi* infection, but no other significant difference was found. One mixed infection with *P. falciparum* was found among each patient group. The RDT technique used showed a low sensitivity (<30%) for detecting *P. ovale* once mixed infections were excluded.

Clinical and therapeutic data for the patients are shown in Table 3. All 3 asymptomatic patients had *P. ovale curtisi* infection; 1 infection was detected as an incidental finding in a blood smear for sickle cell disease, and the other 2 were found during studies of anemia after negative results were found by thick film examination and RDT results. The remaining 7 patients with negative thick smear and positive PCR results reported at least fever. Symptomatic patients showed no difference in clinical signs and symptoms, but those with *P. ovale wallikeri* did have a higher mean number of symptoms (3.5 per patient) than did those with *P. ovale curtisi* (2.7). The number of complications was similar in both groups; 3 cases of severe anemia occurred, 2 of them related to sickle cell disease, and 1 case of ADRS occurred in a patient with *P. ovale wallikeri* infection.

Most patients were admitted to a hospital and received inpatient treatment. Roughly half the patients in each group

Table 1. Demographic and epidemiologic characteristics of patients with imported *Plasmodium ovale curtisi* or *P. ovale wallikeri* infections, Spain, 2005–2011*

| Characteristic | *P. ovale curtisi*, n = 21 | *P. ovale wallikeri*, n = 14 | p value |
|---|---|---|---|
| Patient sex | | | 0.332 |
| M | 10 (47.6) | 9 (64.3) | |
| F | 11 (52.4) | 5 (35.7 ) | |
| Patient age, y, median (IQR) | 36.50 (23.04–52.66) | 38.33 (11.79–45.27) | 0.377 |
| Age <15 | 3 (14.3) | 4 (28.6) | 0.401 |
| Ethnicity | | | 0.721 |
| Black | 15 (71.4) | 9 (64.3) | |
| White | 6 (28.6) | 5 (35.7) | |
| Type of patient | | | 0.260 |
| Early immigrant | 6 (28.6) | 4 (28.6) | |
| Traveler | 14 (66.7) | 10 (71.4) | |
| Reason for travel | | | |
| Visiting friends and relatives | 9 (42.8) | 7 (50.0) | |
| Tourism | | 1 (7.1) | |
| Work | 3 (14.3) | 2 (14.3) | |
| Cooperation | 2 (9.5) | | |
| Unknown | 1 (4.8) | | |
| Duration of travel, d, median (IQR) | 75 (23.25–91.50) | 23 (15.00–81.50) | 0.279 |
| Country of infection | | | 0.486 |
| Equatorial Guinea | 12 (57.1) | 7 (50.0) | |
| Nigeria | 2 (9.5) | 3 (21.4) | |
| Equatorial Guinea or Cameroon | 1 (4.8) | 0 | |
| Ghana | 1 (4.8) | 1 (7.1) | |
| Ethiopia | 1 (4.8) | 0 | |
| Guinea-Conakry | 1 (4.8) | 0 | |
| Liberia | 1 (4.8) | 0 | |
| Angola | 1 (4.8) | 0 | |
| Guinea-Bissau | 1 (4.8) | 0 | |
| Guinea-Conakry or Senegal | 0 | 1 (7.1) | |
| Côte d'Ivoire | 0 | 1 (7.1) | |
| Mozambique | 0 | 1 (7.1) | |
| Chemoprophylaxis | | | 0.627 |
| No prophylaxis | 17 (81.0) | 13 (92.9) | |
| Mefloquine, incomplete | 1 (4.8) | 1 (7.1) | |
| Mefloquine | 1 (4.8) | 0 | |
| Doxycycline | 1 (4.8) | 0 | |
| Atovaquone/proguanil | 1 (4.8) | 0 | |
| Days from arrival to onset of symptoms, median (IQR) | 94.5 (12.5–297.2) | 9.5 (2.7–58.2) | 0.077 |
| Days from onset of symptoms to diagnosis, median (IQR) | 8 (2.7–16.5) | 3.5 (2.0–7.7) | 0.206 |
| Recent *Plasmodium* infection | 3 (14.3) | 3 (21.4) | >0.999 |
| Other infections | | | |
| Hepatitis B virus | | | >0.999 |
| Active | 1/11 (9.1) | 0/10 | |
| Cured or vaccinated | 6/11 (54.5) | 5/10 (50.0) | |
| Negative | 4/11 (36.4) | 5/10 (50.0) | |
| Hepatitis C virus | 1/7 (14.3) | 0/10 | 0.412 |
| HIV | 1/7 (14.3) | 0/10 | 0.412 |
| Filariasis† | 3/6 (50.0) | 0/4 | 0.200 |
| Intestinal parasites‡ | 3/6 (50.0) | 1/4 (25.0) | 0.571 |
| Other underlying conditions | 9 (42.8) | 6 (42.8) | >0.999 |
| Diabetes mellitus | 2 (9.5) | 1 (7.1) | |
| Drepanocytosis | 2 (9.5) | 0 | |
| Hypertension | 4 (19.0) | 2 (14.3) | |
| Obesity | 1 (4.8) | 0 | |
| Acute pancreatitis | 0 | 1 (7.1) | |
| Policystosis and nephrectomy | 0 | 1 (7.1) | |
| Oligoarthritis | 0 | 1 (7.1) | |
| Glucose-6-phosphate dehydrogenase deficiency | 2/14 (14.3) | 0/8 | 0.515 |
| Pregnancy | 1 (4.8) | 0 | >0.999 |

*Values are no. (%) patients or no. positive/total no. (%) patients unless otherwise indicated. IQR, interquartile range.
†*Mansonella perstans* (n = 2), *Loa loa* (n = 1).
‡*Trichiuris trichiura* (n = 3), hookworms (n = 2), *Ascaris lumbricoides* (n = 2), *Strongyloides stercoralis* (n = 1), *Entamoeba hystolitica* (n = 1).

Table 2. Microbiological characteristics of patients with imported *Plasmodium ovale curtisi* or *P. ovale wallikeri* infections, Spain, 2005–2011*

| Characteristic | *P. ovale curtisi*, n = 21 | *P. ovale wallikeri*, n = 14 | p value |
|---|---|---|---|
| Positive thick smear, no. (%) patients | 16 (76.2) | 10 (71.4) | >0.999 |
| Positive by PCR only, no. (%) patients | 5 (23.8) | 4 (28.6) | >0.999 |
| Parasitemia, μL | 2,800 (773.25–5,484.25) | 1,243.50 (337.75–6,200.00) | 0.699 |
| Mixed infection, no. (%) patients | 1† (4.8) | 1† (7.1) | >0.999 |
| Rapid diagnostic test result, no. positive/total no. patients (%) | | | |
| Common antigen positive | 4/16 (25.0) | 4/12 (33.3) | 0.691 |
| *P. falciparum* antigen positive | 1/15 (6.7) | 2/12 (16.6) | 0.569 |
| Leukocyte count, $\times 10^9$ cells/L | 7.2 (4.9–8.7) | 5.5 (4.2–8.2) | 0.309 |
| Hemoglobin, g/dL | 11.6 (9.7–13.6) | 10.9 (9.6–12.1) | 0.364 |
| Platelet count, $\times 10^9$ cells/L | 126 (106.0–182.5) | 91.5 (54.7–117.7) | **0.031** |
| Albumin, g/dL | 3.7 (3.3–4.1) | 3.4 (2.8–3.7) | 0.063 |
| Creatinine, mg/dL | 0.88 (0.6–1.1) | 0.97 (0.5–1.1) | 0.730 |
| Lactate dehydrogenase, IU/L | 434.5 (358.7–807.7) | 563 (462.5–731.7) | 0.200 |
| Aspartate aminotransferase, IU/L | 24.5‡ (20.0–40.2) | 31 (22–41) | 0.624 |
| Alanine aminotransferase, IU/L | 25.5‡ (16.0–49.7) | 23 (18.5–47.0) | 0.785 |
| Total bilirubin level, mg/dL | 0.68‡ (0.6–1.2) | 0.87 (0.6–1.4) | 0.426 |

*Values are median (interquartile range) unless otherwise indicated. Boldface indicates significance.
†*P. falciparum* was second infection for both patients.
‡One patient had active hepatitis B virus infection.

received chloroquine alone. Almost all patients showed good tolerance to treatment and favorable clinical evolution. More than a quarter (33.3% of *P. ovale curtisi* and 28.6% of *P. ovale wallikeri*) did not receive primaquine for radical cure, 2 because of glucose-6-phosphate dehydrogenase deficiency. One patient was lost to follow-up and did not receive any treatment or monitoring.

## Discussion

Our comparative study of the epidemiologic and clinical characteristics of patients with *P. ovale curtisi* and *P. ovale wallikeri* infection found only 1 statistically significant result, a higher rate of severe thrombocytopenia among patients with *P. ovale wallikeri* infection (p = 0.031). Nevertheless, we noted nonsignificant results, including a shorter time from arrival to onset of symptoms in travelers who acquired *P. ovale wallikeri* infection (p = 0.077). This finding fits with findings from a recently published larger series from the United Kingdom in which a significantly shorter latency was found for *P. ovale wallikeri* compared with *P. ovale curtisi* infection (*20*). We also found a trend toward a shorter stay in Africa and shorter interval between onset of symptoms and diagnosis among patients with *P. ovale wallikeri* infection, which could reflect easier transmission, shorter latency, or higher relapse rates. This finding might also mean that slightly more severe illness, including higher median fever (39.7°C vs. 38.4°C) and a greater number of symptoms (3.5 vs. 2.7 per symptomatic patient), led more patients with *P. ovale wallikeri* infection than those with *P. ovale curtisi* infection to seek medical attention earlier. However, the higher percentage of travelers who took at least partial prophylaxis among the *P. ovale curtisi* group (19.2%) might also explain a longer time of onset. A high frequency of nausea and vomiting was found among those with *P. ovale wallikeri* infection, a symptom that was absent among those with *P. ovale curtisi* infection. However, because most cases were asymptomatic or had mild to moderate clinical features, finding significant differences within this narrow clinical spectrum may be especially difficult and might require a much larger study.

Because the time of infection is more likely to be accurately known, patients with imported malaria might make a better group for study of the epidemiologic and clinical characteristics of different *Plasmodium* species than those living in malaria-endemic countries. Moreover, signs or symptoms may be less affected by other tropical infections, including mixed *Plasmodium* infections, or by patient immunity; imported malaria occurs among a larger number of nonimmune patients and patients who are visiting friends and relatives. Differentiation among primary infection or relapse continues to be practically impossible, but the longer the time of latency, the more probable a relapse.

Although criteria for admission to each hospital involved in the study were different, *P. ovale wallikeri* patients were admitted more frequently. The number of complications was similar in both groups; 3 cases of severe anemia were reported, 2 related to sickle cell disease, and 1 case of ADRS occurred in a patient infected with *P. ovale wallikeri* (*8*). Sickle cell trait was described as a risk factor for infection with *P. ovale* in Senegal (*21*), and a recent series showed 3 of 16 patients with *P. ovale* infection were homozygous for sickle cell disease (*22*), a clearly disproportionate number.

Regarding symptomatic patients, the Bangladesh study (*5*) showed a trend toward a larger number of asymptomatic *P. ovale curtisi* infections (90% vs. 75% for *P. ovale wallikeri*), which would be consistent with our results. In that study, all 13 mixed infections of *P. ovale* with other

Table 3. Clinical and therapeutic characteristics of patients with imported *Plasmodium ovale curtisi* or *P. ovale wallikeri* infections, Spain, 2005–2011*

| Characteristic | *P. ovale curtisi*, n = 21 | *P. ovale wallikeri*, n = 14 | p value |
|---|---|---|---|
| Asymptomatic | 3 (14.3) | 0 | 0.259 |
| Fever | 18 (85.7) | 14 (100.0) | 0.259 |
| Tertian fever | 1 (4.8) | 3 (21.4) | 0.279 |
| Maximum temperature, ºC, median (IQR) | 38.4 (37.5–40.0) | 39.7 (38.9–40.5) | 0.088 |
| Chills | 3 (14.3) | 3 (21.4) | 0.664 |
| Sweating | 0 | 1 (7.1) | 0.400 |
| Headache | 6 (28.6) | 4 (28.6) | >0.999 |
| Nauseas | 0 | 3 (21.4) | 0.056 |
| Vomitus | 0 | 3 (21.4) | 0.056 |
| Astenia | 2 (9.5) | 3 (21.4) | 0.369 |
| Epigastralgia | 2 (9.5) | 0 | 0.506 |
| Arthralgia | 5 (23.8) | 3 (21.4) | >0.999 |
| Myalgia | 6 (28.6) | 4 (28.6) | >0.999 |
| Diarrhea | 1 (4.8) | 1 (7.1) | >0.999 |
| Chest pain | 1 (4.8) | 1 (7.1) | >0.999 |
| Cough | 4 (19.0) | 3 (21.4) | >0.999 |
| Dyspnea | 0 | 1 (7.1) | 0.400 |
| Dizziness | 2 (9.5) | 0 | >0.999 |
| Splenomegaly | 5 (23.8) | 3 (21.4) | >0.999 |
| Complications or severe malaria | 2 (9.5) | 2 (14.3) | >0.999 |
| Hemolytic crisis | 1 (4.8) | 0 | |
| Severe anemia, hemoglobin <7 g/dL | 1 (4.8) | 1 (7.1) | |
| Acute respiratory distress syndrome | 0 | 1 (7.1) | |
| Admission to hospital | 13 (61.9) | 13 (92.9) | 0.056 |
| Duration of hospitalization, d, median (IQR) | 4 (3.0–7.5) | 5 (3.5–7.5) | 0.390 |
| Treatment | | | 0.563 |
| Chloroquine | 12 (57.1) | 7 (50.0) | |
| Other treatment | 8 (38.1) | 7 (50.0) | |
| Quinine + doxycycline | 3 (14.3) | 4 (28.6) | |
| Atovaquone/proguanil | 3 (14.3) | 1 (7.1) | |
| Quinine + clindamycin + chloroquine/proguanil | 1 (4.8) | 0 | |
| Quinine + clindamycin + chloroquine | 0 | 1 (7.1) | |
| Mefloquine | 0 | 1 (7.1) | |
| Atovaquone/proguanil + chloroquine | 1 (4.8) | 0 | |
| No treatment | 1 (4.8) | 0 | |
| Primaquine | 14 (66.7) | 10 (71.4) | >0.999 |
| Compliance | 19/21 (90.5) | 13/13 (100.0)† | 0.513 |

*Values are no. (%) patients or no. positive/total no. (%) patients unless otherwise indicated. IQR, interquartile range.
†One patient was lost to follow-up.

*Plasmodium* species were asymptomatic; in our study, the 2 patients who had mixed infections with *P. falciparum* had at least fever. The number of *P. ovale* monoinfections in our study confirmed by PCR is high, unlike the number in malaria-endemic areas, where most infections are mixed (*5*,*7*,*23*) and a high rate of submicroscopic carriage occurs (*24*). In part, our finding may result from previous antimalarial treatment or prophylaxis in a number of patients, which could have minimized the number of *Plasmodium* parasites in the blood.

Among the laboratory results, the only significant difference was found in platelet count, with more severe thrombocytopenia seen among patients with *P. ovale wallikeri* infection than among those with *P. ovale curtisi* infection. Thrombocytopenia is a common finding in patients with malaria; a previous series found 10 (66.6%) of 15 patients with imported *P. ovale* infection had platelet counts of <140,000/mL (*22*). The mechanisms that produce thrombocytopenia in malaria are not known but seem related to a greater severity of illness (*25*). Some studies also suggest an inverse correlation between the level of parasitemia and platelet count (*26*).

We found that indirect parameters of hemolysis, such as hemoglobin, lactate dehydrogenase, and bilirubin levels, were less impaired among patients with *P. ovale curtisi* infection than those with *P. ovale wallikeri* infection, even including 2 patients with sickle cell disease, who had more severe anemia. Albumin values, diminished by other multiple types of infections, also tend to be lower in patients with *P. ovale wallikeri* infection. For transaminases, if we were to exclude a patient with chronic hepatitis B and hypertransaminasemia, we would also find higher values for patients with *P. ovale wallikeri* infection. These data collectively raise the hypothesis that *P. ovale wallikeri* is slightly more pathogenic, which warrants further investigation.

Parasitemia levels were not significantly different between the 2 groups (p = 0.699). In 2 recent studies of *P. ovale* infection that included determination of parasitemia

levels and genetic analysis (*5*,*15*), no differences were described. Case reports of high parasitemia levels in patients with *P. ovale wallikeri* infection in Southeast Asia are isolated and noncomparative (*12*–*14*).

Sooner and better access to health care in industrialized countries might provide a broader range of diagnostic tools for malaria, including RDT, thick and thin film examination, or PCR. Current techniques of RDT still show a low sensitivity for detecting *P. ovale* (*27*,*28*). This problem is usually explained by the genetic variability of the 2 subspecies and the low levels of parasitemia detected. After discarding mixed infections, in our study, the aldolase-based RDT used as a common antigen of *Plasmodium* obtained 20% sensitivity for detection of *P. ovale curtisi* and 27.27% for *P. ovale wallikeri*. Results are poorer than those recently published by Bauffe et al., who found a higher false-negative rate of infection for *P. ovale curtisi* than for *P. ovale wallikeri* (60% vs. 43%, respectively) and no significant differences in parasitemia levels (*15*).

Although circulation of *P. ovale* was known in these countries, our study provides PCR confirmation for *P. ovale curtisi* infections from Guinea-Conakry, Ethiopia, and Liberia and *P. ovale wallikeri* infections from Mozambique. Both species have been described as sympatric in time and space in Equatorial Guinea, Republic of Congo, Uganda, Bangladesh, and Angola (*5*,*11*,*29*), maintaining genetic differentiation, which supports the hypothesis of 2 distinct subspecies. Moreover, 2 cases of *P. ovale curtisi* and *P. ovale wallikeri* co-infection have been reported (*5*,*29*).

We found a high rate of underlying chronic diseases among the patients in this study, especially homozygous sickle cell anemia and diabetes mellitus. Previous studies have shown that carriage of the sickle cell trait confers increased susceptibility to *P. ovale* infection (*20*) and that diabetes and HIV infection confer increased susceptibility to *P. falciparum* infection (*30*).

In industrialized countries, improved clinical and microbiological control after treatment and radical cure with primaquine can be achieved for malaria. *P. ovale* seems to remain sensitivity to chloroquine and other antimalarial drugs. Patients in our study showed good clinical response that could be followed up without any relapse, including among those who had complications. The treatment differences reflect the different hospital managing protocols, patient age and pregnancy status, or difficulty in identifying *P. ovale* initially.

Our study has limitations. First, the small number of patients may lack sufficient statistical power to show differences between infections with different *Plasmodium* species. Second, the low performance of genetic amplification may have caused some sample selection bias (e.g., a higher number of samples that were stored short term or came from patients with higher parasitemia levels). Third, patient selection was not systematically planned but was done on the basis of decisions of physicians from many hospitals who sent samples to the reference laboratory and subsequently agreed to participate in this study. Fourth, the study's retrospective design led to gaps in the information collected. Fifth, only strains of *P. ovale* from Africa were analyzed, and patients were from Africa and Europe; a study of infections and patients from Asia or Oceania might show different results. Last, more diversity in the geographic origin of the strains and a mix of nonimmune and semiimmune patients would lead to more heterogeneous study groups.

In summary, after comparing epidemiologic, clinical, and analytic data for patients with *P. ovale wallikeri* and *P. ovale curtisi* infections, we found significantly more marked thrombocytopenia among patients with *P. ovale wallikeri* infection, but we found no other significant differences. However, some trends toward slightly greater pathogenicity were observed for *P. ovale wallikeri* infection. The description of both genotypes occurring in sympatry without hybrid forms in an increasing number of countries supports the idea of 2 well-defined species. Larger prospective studies should be conducted to more fully explore this hypothesis.

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# *Coxiella burnetii* Seroprevalence and Risk for Humans on Dairy Cattle Farms, the Netherlands, 2010–2011

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Q fever, caused by *Coxiella burnetii*, is a recognized occupational infection in persons who have regular contact with ruminants. We determined *C. burnetii* seroprevalence in residents living or working on dairy cattle farms with ≥50 adult cows and identified risk factors for seropositivity. Serum samples from farm residents, including employees, were tested for *C. burnetii* IgG and IgM; seroprevalence was 72.1% overall and 87.2%, 54.5%, and 44.2% among farmers, spouses, and children, respectively. Risk factors included farm location in southern region, larger herd size, farm employment, birds in stable, contact with pigs, and indirect contact with rats or mice. Protective factors included automatic milking of cows and fully compliant use of gloves during and around calving. We recommend strengthening general biosecurity measures, such as consistent use of personal protective equipment (e.g., boots, clothing, gloves) by farm staff and avoidance of birds and vermin in stables.

Q fever is an occupational zoonosis caused by *Coxiella burnetii*, a gram-negative bacterium (*1*). Ruminant farmers, laboratory workers, dairy workers, and veterinarians are at particular risk for infection. Humans usually acquire Q fever by inhalation of *C. burnetii* aerosolized from contaminated materials originating from infected animals. The primary animal reservoirs responsible for human infections are cattle, sheep, and goats, which can shed *C. burnetii* in urine, feces, milk, and birth products. Before 2007, the seroprevalence of *C. burnetii* antibodies within the general population of the Netherlands was 2.4%; keeping ruminants and increasing age were risk factors for seropositivity (*2*). During 2007–2009, Q fever was a major public health problem in the Netherlands; >4,000 human cases were reported (*3*). Large-scale interventions primarily targeting small ruminants were used to control the epidemic. In 2008, mandatory vaccination was conducted in a defined cluster area and later nationwide. In 2009–2010, a program was implemented to cull pregnant dairy goats and sheep on farms with *C. burnetii*–positive animals identified through a national bulk tank milk (BTM) screening (*4*). Since then, the incidence of acute Q fever cases has diminished substantially (*5*), but chronic cases still occur (*6*). No epidemiologic associations between Q fever cases in humans and dairy cattle were identified during this epidemic, nor have any been described in other Q fever outbreaks (*7*). Nevertheless, recent reports indicate that *C. burnetii* is widespread among Dutch dairy cattle herds (prevalence 78.6% [ELISA] or 56.6% [PCR] in BTM samples) (*8*). In 2008, seroprevalence was 16.0% in lactating cows and 1.0% in young animals (*8*).

*C. burnetii* seroprevalence estimates for dairy cattle farm residents in the Netherlands are outdated, and risk factors associated with seropositivity are seldom studied. This lack of data inhibits accurate assessment of the public health risk. To inform control measures and provide advice for persons living/working on a dairy cattle farm (DCF), we conducted a cross-sectional study to investigate the seroprevalence of *C. burnetii* antibodies in DCF residents/workers and identified participant-based and farm-based risk factors for seropositivity. The study was approved by the Medical Ethics Committee of the University Medical Centre Utrecht (no. 09–189/K).

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## Methods

A total of 3,000 DCFs housing ≥50 adult dairy cows were randomly selected for possible participation in the study from a national database maintained by the Animal Health Service. In September and November 2010, information and recruitment materials were sent to 1,000 and 2,000 farms, respectively. Farms were enrolled in the study after returning a completed informed-consent form. After 4 weeks, nonresponding farms from the first mailing received a written reminder. Nonresponding farms from the second mailing did not receive a reminder because the goal of enrolling 296 farms had been reached; this number was determined on the basis of power calculations assuming 50.0% prevalence and 5.5% precision. We contacted enrolled farms by telephone to confirm participation and determine the number of participants. Dairy cattle farmers and up to 2 family members or farm employees ≥12 years of age were eligible for participation in the study. Participants completed a questionnaire about personal characteristics (e.g., age, medical history, farm-related activities, contact with livestock and companion animals, consumption of unpasteurized dairy products, and use of personal protective equipment [PPE]) and provided a serum sample (collected by a laboratory assistant during a home visit). The farm owner or manager completed a questionnaire about herd size, cattle housing, presence of other livestock and companion animals, farm facilities, animal health, and hygiene measures. Participating farms were requested to provide one BTM sample for testing by ELISA and PCR, as described (*8*).

### Serology

We used an immunofluorescence assay (IFA) (Focus Diagnostics, Cypress, CA, USA) to test serum samples for *C. burnetii* phase I and II IgM and IgG. All samples were screened at an initial dilution of 1:32; those with negative results were considered negative. Positive samples were further classified as indicative of relatively recent infections (IgM phase II titer ≥32) or past infections (IgG phase II titer ≥32 and IgM phase II titer <32). Samples with all other outcomes were considered negative. The term relatively recent was chosen because phase II IgM is commonly found up to 1 year after infection in acute Q fever cases, but it may persist up to 3 years (*9*). Phase I and II IgG end point titers were determined for all seropositive persons. In agreement with chronic Q fever diagnostic criteria used in the Netherlands (*10*), phase I IgG titers ≥1,024 in samples in the past infection group were considered indicative of possible chronic infection.

### Data Analysis

Participating and nonparticipating farms were compared with respect to herd size; distance to nearest *C. burnetii*–positive BTM small-ruminant farm; goat, sheep, and cattle density; location by province and region; and degree of urbanization. We used the Mann-Whitney U test to determine differences in continuous variables and the $\chi^2$ test to analyze categorical variables. We performed univariate logistic regression analyses to determine the main factors associated with *C. burnetii* seropositivity among participants ($p<0.20$, likelihood ratio test). Potential farm-based risk factors were analyzed by univariate multilevel analyses; a unique farm identifier was used as the cluster variable. Distributions of continuous variables were studied, and variables not linearly related to the outcome variable were categorized on the basis of biological arguments (e.g., nearest *C. burnetii*–positive BTM small-ruminant farm) or, if those were lacking, on medians (e.g., goat density within 5-km radius). Participant age was always kept in the model because of its frequent relation with seropositivity. Variables with <10.0% of participants in a risk category were excluded from further analysis. If several variables were found interrelated in the univariate analysis, only the most informative and relevant variable was selected for inclusion.

Risk factors determined to be significant ($p<0.20$) in univariate analyses of the participant-based and farm-based data were incorporated into multivariate logistic regression and multivariate multilevel analyses, respectively. Stratified multivariate analyses for participant risk factors were performed separately for farmers and for the remaining group. Starting with a full model, manual backward elimination was performed; all variables meeting the 10.0% significance level in the likelihood ratio test were kept in the final model. Two-way interactions between biologically plausible variables in the multivariate model were investigated. Last, variables included in the final multivariate model for participant-based factors and those included in the multilevel model for farm-based factors were combined in a multivariate multilevel analysis to identify the independent risk determinants for seropositivity. The final model fit was assessed by the quasi-likelihood under the independence model criterion goodness-of-fit statistic for generalized estimation equation models. SAS version 9.2 (SAS Institute, Cary, NC, USA) was used for all analyses.

## Results

### Nonresponse Analysis

Of the 3,000 invited farms, 311 provided a BTM sample, and 755 persons from 309 (10.3%) farms participated in this study by providing a serum sample. A farm-based questionnaire was available for 736 (97.5%) persons from 301 farms, and a participant-based questionnaire was completed by 729 (96.6%) persons from 308

farms. Compared with nonparticipating farms, participating farms were a median of 1.5 km closer to small ruminant farms with *C. burnetii*–positive BTM samples (Table 1). In addition, the density of sheep within a 5-km radius of participating farms was higher than that for nonparticipating farms; however, the absolute difference was very small (3 sheep/km$^2$).

### Seroprevalence

Overall *C. burnetii* seroprevalence was 72.1% (95% CI 68.8%–75.3%), and seroprevalence among farmers, spouses, and children (12–17 years of age) was 87.2%, 54.5%, and 44.2%, respectively (Table 2). Seroprevalence was univariately significantly higher among male participants, farmers, and participants ≥35 years of age (Table 3, Appendix, wwwnc.cdc.gov/EID/article/20/3/13-1111-T3.htm). The median duration of farm residence was 28 years (range 0–56). IgG phase II end titers were known for 534 (98.9%) of 540 *C. burnetii* IgG phase II–seropositive participants: 32 (n =166), 64 (n = 92), 128 (n = 119), 256 (n = 106), 512 (n = 39), 1,024 (n = 10), 2,048 (n = 1), and 4,096 (n = 1). IgG phase I end titers were known for 283 (97.6%) of the 290 IgG phase I–seropositive participants: 32 (n = 105), 64 (n = 73), 128 (n = 61), 256 (n = 32), 512 (n = 10), 1,024 (n = 1), and 2,048 (n = 1). These last 2 participants, with phase I titers of 1,024 and 2,048, respectively, had lower IgG phase II titers (512 and 1,024, respectively), and according to chronic Q fever diagnostic criteria used in the Netherlands (*10*), these participants met the conditions for possible chronic Q fever infection. We could not confirm that these truly were chronic Q fever cases because clinical information (e.g., presence of vascular infection, endocardial involvement, or other clinical risk factors) was lacking.

Nine (1.2%) participants from 8 farms were classified as having a relatively recent infection (IgM phase II titer range 32–256). All 8 farms were within 2.5–21.2 km of the nearest *C. burnetii*–positive BTM small-ruminant farm, and 4 of the 8 were within 3 km.

Four participants reported having had Q fever diagnosed by a physician during 2008–2010. On the basis of serum samples obtained at study entry, 3 of these participants had a serologic profile indicating past infection. These 3 participants lived in the southern or eastern region of the Netherlands on farms within a 3-km radius of the nearest small-ruminant farm with *C. burnetii*–positive BTM samples. The fourth participant had no serologic evidence of a past infection and lived 14 km from the nearest small-ruminant farm with *C. burnetii*–positive BTM samples.

Table 1. Nonresponse analyses of farms in a study of *Coxiella burnetii* seroprevalence and risk for seropositivity in humans on dairy cattle farms, the Netherlands, September 2010–March 2011

| Variable | Farms | | p value |
|---|---|---|---|
| | Participating, n = 311 | Nonparticipating, n = 2,685 | |
| Categorical, no. (%) | | | |
| Farm located inside vaccination area | 83 (26.4) | 590 (21.9) | 0.08 |
| Farm region* | | | 0.36 |
| North | 80 (25.4) | 781 (29.1) | |
| East | 104 (33.7) | 911 (33.9) | |
| West | 57 (18.7) | 494 (18.3) | |
| South | 70 (22.2) | 503 (18.7) | |
| Degree of urbanization of the farm municipality | | | 0.77 |
| Moderately, strongly, or extremely (>1,000 addresses/km$^2$) | 1 (0.3) | 17 (0.6) | |
| Hardly (500–1,000 addresses/km$^2$) | 10 (3.2) | 94 (3.5) | |
| Not (<500 addresses/km$^2$) | 300 (96.5) | 2,574 (95.9) | |
| Numerical, median no. | | | |
| No. cows in 2008 | | | |
| <1 | 35 | 35 | 0.44 |
| 1–2 | 26 | 26 | 0.65 |
| >2 | 85 | 86 | 0.16 |
| Nearest bulk tank milk positive small-ruminant farm (meters) | 9,793 | 11,301 | 0.01 |
| Goat density (animals/km$^2$)† | | | |
| Within 5-km radius | 9.2 | 6.7 | 0.27 |
| Within 10-km radius | 9.3 | 9.2 | 0.26 |
| Sheep density (animals/km$^2$)† | | | |
| Within 5-km radius | 30 | 33 | 0.04 |
| Within 10-km radius | 34 | 35 | 0.11 |
| Cattle density (animals/km$^2$) within 5-km radius† | | | |
| Including own animals | 178 | 181 | 0.29 |
| Excluding own animals | 175 | 179 | 0.27 |
| Cattle density (animals/km$^2$) within 10-km radius† | | | |
| Including own animals | 170 | 170 | 0.99 |
| Excluding own animals | 169 | 169 | 0.91 |

*North represents Groningen, Friesland, and Drenthe Provinces; East represents Gelderland, Overijssel, and Flevoland Provinces; West represents Noord–Holland, Zuid–Holland, Utrecht, and Zeeland Provinces; and South represents Limburg and Noord–Brabant Provinces.
†Corrected for area in the Netherlands.

Table 2. Participant characteristics and *Coxiella burnetii* seroprevalence among dairy cattle farm residents, the Netherlands, September 2010– March 2011

| Participant characteristic | Total no. residents/no. positive (%) | 95% CI |
|---|---|---|
| All participants | 755/544 (72.1) | 68.8–75.3 |
| Sex | | |
| M | 431/368 (85.4) | 82.0–88.7 |
| F | 323/176 (54.5) | 49.0–59.9 |
| Age, y | | |
| <35 | 169/107 (63.3) | 56.0–70.7 |
| 35–44 | 176/131 (74.4) | 67.9–80.9 |
| 45–54 | 252/185 (73.4) | 67.9–78.9 |
| ≥55 | 132/106 (80.3) | 73.4–87.2 |
| Role | | |
| Farmer | 361/315 (87.2) | 83.8–90.7 |
| Spouse | 222/121 (54.5) | 47.9–61.1 |
| Child <18 y | 52/23 (44.2) | 30.3–58.2 |
| Child ≥18 y | 54/40 (74.1) | 62.0–86.1 |
| Other* | 40/30 (75.0) | 61.0–89.0 |

*Represents other family members and employees.

### Univariate Analyses at Participant and Farm Levels

Risk factors for seropositivity for farmers/workers and residents included age ≥35 years; farm employment; directly performing cattle-related tasks; contact with cattle, pigs, hay, cattle food, raw milk, manure, or cattle birth products; presence of rats or mice on the farm; and growing up on a farm (Table 3, Appendix). Protective factors included poultry and compost contact and fully compliant use of gloves during and around calving. Farm-based risk factors included a larger herd size, farm location in the southern region, an annual peak in calving, having beef cattle on the farm, and the presence of birds in the stable. Protective factors included automatic milking, having pet cats or rabbits, and having farm clothes and boots available for professional visitors (e.g., veterinarians and feed specialists) (Table 4). No relationship was found between PCR or ELISA status on the basis of BTM samples and participant seropositivity.

### Multivariate and Multilevel Analyses

Of the 21 variables considered in the multivariate participant model, 8 were independently associated with seropositivity: age ≥55 years; working on the farm; fully compliant use of gloves during cattle birth care; contact with pigs, cattle at other farms, poultry, or compost; and indirect contact with rats or mice (Table 5). Interaction terms did not improve the model.

Of the 9 variables considered in the multilevel farm model, 6 were independently associated with seropositivity; larger herd size, farm location in the southern region, beef cattle on the farm, use of food concentrate, and presence of birds in the stable were risk factors, and automatic milking was a protective factor (Table 6). In the combined multilevel analysis, the 12 significant factors from the multivariate participant and multilevel farm models, in addition to age, were combined in 1 model. The nonstratified model had a clearly better fit than the stratified model for farmers. Farm location within 8 km of the nearest *C. burnetii*–positive BTM small-ruminant farm (odds ratio 2.3, 95% CI 1.2%–2.5%) was a risk factor in the final stratified multilevel model among farmers and was therefore included in the combined multilevel analysis. In the final overall model, independent risk factors were age ≥55 years, farm employment, pig contact, larger herd size, farm location in the southern region, beef cattle on the farm, cattle contact at other farms, and presence of birds in the stable. Indirect contact with rats or mice was borderline significant (Table 7). Protective factors were contact with poultry or compost, use of automatic milking, and fully compliant use of gloves during birth care. We ran an additional model by adding a protective variable (farm clothes and boots available for professional visitors), as described in Table 5, in the farm-based and combined multilevel models. Doing so resulted in a final model with the same factors as shown in Table 7, except that automatic milking was replaced by another protective factor (farm clothes and boots available for professional visitors) and 2 borderline significant risk factors (distance to the nearest *C. burnetii*–positive BTM small-ruminant farm and use of by-product feedstuffs) (data not shown).

## Discussion

The overall seroprevalence of 72.1% among DCF residents, including employees, was high, indicating a considerable lifetime risk for acquiring *C. burnetii* infection. Seroprevalence was highest among farmers (87.2%). The observed seroprevalence was similar to that determined by a study from the 1980s that showed an estimated seroprevalence of 68.0% among 94 Dutch dairy farm residents; however, laboratory methods used in that study were different than those used by us (*11*). The 72.1% seroprevalence was also compatible with recent estimates among dairy goat farms residents (68.7%) (*12*), dairy sheep farms residents (66.7%) (*13*), and livestock veterinarians (65.1%) (*14*). Estimates for these livestock-associated groups

Table 4. Univariate logistic model of farm-based characteristics associated with *Coxiella burnetii* positivity among dairy cattle farm residents, the Netherlands, September 2010–March 2011*

| Variable | No. residents total (% positive) | OR (95% CI) |
|---|---|---|
| No. cows on farm in 2008†‡ | 755 (72.1) | 1.0 (1.0–1.0) |
| Nearest bulk tank milk positive small-ruminant farm† | | |
| <8 km | 331 (75.8) | 1.4 (1.0–1.9) |
| ≥8 km | 424 (69.1) | Reference |
| Municipal cattle density, including beef calves§ | 755 (72.1) | 1.0 (1.0–1.0) |
| Farm location | | |
| Inside small-ruminant vaccination area | 202 (78.2) | 1.6 (1.0–2.3) |
| Outside small-ruminant vaccination area | 553 (69.8) | Reference |
| Farm region† | | |
| South | 170 (80.6) | 1.8 (1.2–2.7) |
| Other | 585 (69.6) | Reference |
| Beef cattle on the farm† | | |
| Yes | 79 (82.3) | 1.9 (1.1–3.4) |
| No | 652 (70.7) | Reference |
| Annual peak in calving | | |
| Yes | 135 (76.3) | 1.3 (0.9–2.0) |
| No | 601 (71.1) | Reference |
| Automatic milking† | | |
| Yes | 154 (65.6) | 0.7 (0.5–1.0) |
| No | 580 (73.8) | Reference |
| Use of bedding in stables | | |
| Yes | 717 (72.4) | 1.9 (1.2–2.9) |
| No | 19 (57.9) | Reference |
| Pet cat | | |
| Yes | 444 (69.1) | 0.6 (0. 5–0.9) |
| No | 285 (77.9) | Reference |
| Pet rabbit | | |
| Yes | 202 (64.4) | 0.6 (0.4–0.8) |
| No | 527 (75.7) | Reference |
| Birds in stable† | | |
| Yes | 90 (82.2) | 1.9 (1.0–3.6) |
| No | 644 (70.5) | Reference |
| Use of by-product feedstuffs† | | |
| Yes | 229 (77.3) | 1.5 (1.0–2.1) |
| No | 507 (69.6) | Reference |
| No. cows that calved in 2009‡ | 720 (71.8) | 1.0 (1.0–1.0) |
| No. live-born calves | | |
| <78 | 335 (69.0) | Reference |
| ≥78 | 344 (74.1) | 1.3 (0.9–1.8) |
| No. twin calves | | |
| 1–2 | 272 (69.9) | Reference |
| ≥3 | 313 (76.4) | 1.4 (1.0–2.0) |
| Type of farm management† | | |
| Closed herd | 515 (73.4) | Reference |
| Purchase of cattle | 213 (68.1) | 0.8 (0.6–1.1) |
| No. cattle purchase addresses in 2007† | | |
| 0 or 1 | 649 (72.7) | Reference |
| ≥2 | 76 (64.5) | 0.7 (0.4–1.0) |
| Farm boots and work clothes available for professional visitors | | |
| Yes | 662 (71.3) | 0.7 (0.4–1.1) |
| No | 74 (78.4) | Reference |
| Work clothes available for own personnel | | |
| Yes | 556 (73.6) | 1.4 (1.0–1.9) |
| No | 180 (67.2) | Reference |

*The analysis included the primary farm-based factors associated with positivity (p<0.20 in likelihood ratio test). OR, odds ratio.
†Variable included in later multivariate analysis before manual backward elimination.
‡Risk increases per cow.
§Risk decreases per cow.

exceed the seroprevalence of 2.4% for the Dutch population during the pre-epidemic period, 2006–2007 (*2*), and the seroprevalences of 12.2% and 24.0% among persons residing in the most affected outbreak areas during the epidemic in the Netherlands (*15*,*16*).

Seroprevalence studies of other farmer populations, particularly dairy cattle farmers, are scarce, and, in general, it is difficult to compare international studies because of different study populations, tests, or cutoff values used. However, published seroprevalence estimates are generally

Table 5. Multivariate logistic regression analysis of participant-based characteristics associated with *Coxiella burnetii* positivity among dairy cattle farm residents, the Netherlands, September 2010–March 2011*

| Association with positivity, characteristic | OR (95% CI) |
|---|---|
| Positive association | |
| Age, y | |
| <35 | Reference |
| 35–44 | 1.4 (0.8–2.3) |
| 45–54 | 1.0 (0.6–1.6) |
| ≥55 | 1.9 (1.0–3.5) |
| Work on farm | |
| No | Reference |
| Part time (1–39 h/wk) | 2.4 (1.1–5.2) |
| Full time (≥40 h/wk) | 10.4 (4.2–25.7) |
| Contact with pigs at own or other farm | |
| Yes | 2.6 (1.2–5.4) |
| No | Reference |
| Contact with cows at other farm | |
| Yes | 1.6 (1.0–2.6) |
| No | Reference |
| Indirect contact with rats/mice at own farm | |
| Yes | 1.5 (1.0–2.4) |
| No | Reference |
| Negative association | |
| Use of gloves during cattle birth care | |
| Fully compliant | 0.4 (0.2–0.8) |
| Partly or noncompliant | Reference |
| No birth care | 0.7 (0.4–1.1) |
| Contact with poultry at own farm | |
| Yes | 0.6 (0.4–0.9) |
| No | Reference |
| Contact with compost | |
| Yes | 0.6 (0.3–0.9) |
| No | Reference |

*The analysis included the primary participant-based characteristics associated with positivity (p<0.10 in likelihood ratio test). The number of observations was 712. Model fit was assessed by use of the Hosmer–Lemeshow goodness-of-fit test (p = 0.91). OR, odds ratio.

lower than what we observed. A study using IFA with the same cutoff value that we used estimated a seroprevalence of 27.0% among a UK farm cohort (385 residents/workers) (*17*). Two other studies used a *C. burnetii* phase II IgG ELISA, which is somewhat less sensitive than IFA (*9*), and obtained seroprevalence estimates of 48.8% among Northern Ireland farmers from all types of farms (*18*) and 16.0% among 262 farm residents from 105 DCFs in Germany (*19*). A seroprevalence of 3.0% was observed in 163 residents from 100 farms (most likely cattle or pig) in Denmark; the study used the same IFA that we used, but cutoff values of IgG phase I and II were higher (≥512 and ≥1,024, respectively) (*20*). Using the same cutoff, we would obtain a comparable seroprevalence estimate of 2.7%.

Farm residents living in the southern part of the Netherlands were more likely to be seropositive. This was not surprising because living in the south was a risk factor for dairy goat farmers (*12*). In general, it is possible that seropositive DCF residents were partially affected by the many *C. burnetii*–positive BTM small-ruminant farms nearby. This possibility is supported by the close distance between residential addresses of persons who had a relatively recent infection and nearby *C. burnetii*–positive BTM small-ruminant farms. As determined on the basis of phase II IgM, 1.2% of DCF residents and 11.0% of small-ruminant dairy farm residents had a relatively recent *C. burnetii* infection (*12*,*13*), indicating that the infection among DCF residents was generally in the more distant past. Physicians diagnosed Q fever in 0.5% of DCF residents in our study compared with 4.1% in Dutch goat farm residents (*12*); nevertheless, to ensure a timely diagnosis and treatment, physicians should consider Q fever in patients with compatible symptoms and occupational exposure to cattle (*20*,*21*). In general, clinical illness from *C. burnetii* infection appears to be rare among DCF residents, which fits the suggestion in the literature that cattle-acquired *C. burnetii* infection has a milder clinical course (*20*). In other European countries and the United States, *C. burnetii* infection is endemic in cattle and in humans occupationally exposed to cattle, but there are few clinical cases of acute Q fever (*22*,*23*). A possible explanation is that abortion in late gestation is a key sign of infection in small dairy ruminants, but this is not the case in cattle. *C. burnetii* shedding by cattle is generally lower than that by small ruminants; concomitant and persistent shedding patterns are more frequent in clinically affected cows than healthy ones (*24*–*29*). Furthermore, sheep and goats have seasonal reproduction cycles and generally larger herd sizes, leading to huge amounts of bacteria shed during a short period. Multilocus variable-number tandem-repeat analysis genotyping has indicated that *C. burnetii* genotypes in dairy cattle herds and dairy consumer products (*30*,*31*), except for 1 placenta sample, are clearly distinct from the predominant outbreak genotype found at Dutch small ruminant dairy farms in 2007–2009 (*32*). Upcoming research should elucidate whether the cattle strains circulating in the Netherlands and other countries are less virulent.

Persons ≥55 years of age were at increased risk for seropositivity, which cannot be explained by differences in specific cattle-related tasks, frequency of cattle contact, or hours worked. It may be that host factors or continuous or regular exposure to the bacterium (booster effect) play a role that cannot adequately be assessed through a questionnaire. Full-time farm employment (≥40 h/week) was a risk factor, which corresponds with a study among Dutch livestock veterinarians in which ≥30 hours of weekly animal contact was a risk factor for infection (*14*). Full-time farm employment and working or residing on a dairy (primarily) farm were risk factors in a UK farm cohort (*17*), indicating a dose-response relationship between seropositivity and the number of working hours spent with dairy cattle or in a dairy farm environment in general.

We identified several cattle-related risk factors for seropositivity among cattle farm residents/staff: herd size, cattle

contact at other farms, and presence of beef cattle on their own farm. A larger herd size could pose a risk because of an increased chance for *C. burnetii* introduction or the presence of a larger susceptible population of cows; however, some farm-based risk factors associated with a large herd that were not assessed through the questionnaire might also have caused this effect (*19*,*33*,*34*). Cattle contact at other farms possibly reflects risk from exposure to *C. burnetii* in other infected herds. The presence of beef cattle as a risk factor for DCF residents is not easily explained, but it might reflect risk from more intense birth care and, therefore, more extensive human contact with cattle and birth products.

Protective factors included use of automatic milking and fully compliant use of gloves during birth care. Birth products of *C. burnetii*–infected ruminants are a source of human infections. A German study among veterinarians identified an association between increasing numbers of cattle obstetric procedures performed and seropositivity (*21*). Pig contact, indirect contact with rats/mice, and presence of wild or domesticated birds in the stable were indicated as risk factors in our study. Studies among veterinarians in the Netherlands and the United States identified swine contact as a risk factor (*14*,*35*); however, *C. burnetii* has not been found in pigs in the Netherlands (*30*). Rats and wild birds were identified as *C. burnetii* reservoirs in several studies (*36*–*38*) and as reservoirs on cattle farms in the Netherlands (*39*).

Fully compliant use of gloves during birth care can help farmers protect themselves against *C. burnetii* infection (*21*). Consistent use of farm boots and working clothes for professional visitors was a protective factor in our additional multilevel model. It might appear that the use of protective clothing by visitors will prevent *C. burnetii* transmission to the visitor rather than the farmer; however, providing gloves and farm clothes for visitors indicates a state of optimal awareness on the farm with regard to communicable diseases. In addition, automatic milking of cows might reflect less direct cattle exposure, especially through avoiding contact with the udders, raw milk, manure, and genital fluids, and thus might limit the chance of infection. Statistical analyses indicated lower risk for seropositivity among farm residents exposed to poultry and to compost. We have no biologically plausible explanation for this finding, and the statistical effect might have occurred by chance. Raw milk consumption was a risk factor for seropositivity in German dairy cattle farmers (*19*). Although consumption of raw milk was not an independent risk factor in our study, 21.8% of farm residents reported daily drinking of raw milk. *C. burnetii* exposure during nonautomatic milking could still implicate the risk of inhaling contaminated aerosols during pretreatment of the cow or during accidental raw milk ingestion.

Table 6. Multilevel analysis of farm-based characteristics as independent factors associated with *Coxiella burnetii* positivity among dairy cattle farm residents, the Netherlands, September 2010–March 2011*

| Variable | OR (95% CI) |
|---|---|
| No. cows on farm in 2008† | 1.0 (1.0–1.0) |
| Farm region | |
| South | 1.8 (1.2–2.8) |
| Other | Reference |
| Beef cattle on farm | |
| Yes | 1.7 (1.0–2.8) |
| No | Reference |
| Automatic milking | |
| Yes | 0.7 (0.4–1.0) |
| No | Reference |
| Birds in stable | |
| Yes | 2.0 (1.1–3.8) |
| No | Reference |
| Use of by-product feedstuffs | |
| Yes | 1.4 (1.0–2.0) |
| No | Reference |

*The analysis included the primary farm-based characteristics associated with positivity ($p<0.10$ in likelihood ratio test). The number of observations was 716; the number of levels used was 309 (quasi-likelihood under the independence model criterion 832.88). OR, odds ratio.
†Risk increased per cow.

The relatively low response rate of 10.4% in this study can be explained by a general lack of motivation or awareness among cattle farmers because Q fever was mainly considered a problem among small-ruminant dairy farms. A general fear of consequences resulting from possible control measures targeting the cattle sector comparable with implemented control measures for Q fever in the small-ruminant sector might also have played a role. Study results are, however, considered representative for the Dutch dairy cattle sector because participating and nonparticipating farms were generally comparable.

The overall *C. burnetii* seroprevalence of 72.1% among DCF residents is high. Multilevel analysis identified several plausible risk factors (e.g., employment on a farm, larger herd size, and cattle contact at other farms). A farm location in the southern region as risk factor suggests *C. burnetii* transmission from small-ruminant dairy farms to cattle farm residents living nearby. Use of automatic milking and fully compliant use of gloves during birth care are plausible protective factors, indicating less direct contact with cattle and, thus, a reduced chance of animal-to-human transmission. The dairy cattle sector must inform farmers about potential sources of infection. Biosecurity measures are warranted; for example, wild birds and vermin should be kept out of stables, and farmers/staff should be educated regarding the consistent use of PPE, such as wearing gloves during birth assistance and invasive procedures. Physicians should consider Q fever in the differential diagnosis for dairy cattle farmers with compatible symptoms. Future studies should more explicitly assess the clinical effect of acute and chronic Q fever in humans who live or work on DCFs.

Table 7. Combined multilevel analysis of participant- and farm-based characteristics associated with *Coxiella burnetii* seropositivity in dairy cattle farm residents, the Netherlands, September 2010–March 2011*

| Variable | OR (95% CI) |
|---|---|
| Age, y | |
| <35 | Reference |
| 35–44 | 1.3 (0.8–2.3) |
| 45–54 | 1.2 (0.7–2.0) |
| ≥55 | 1.9 (1.1–3.5) |
| Work on farm | |
| No | Reference |
| Part time (1–39 h/wk) | 2.5 (1.1–5.6) |
| Full time (≥40 h/wk) | 10.7 (4.2–27.0) |
| Use of gloves during cattle birth care | |
| Fully compliant | 0.4 (0.2–0.8) |
| Partly or noncompliant | Reference |
| No birth care | 0.7 (0.4–1.1) |
| Contact with pigs at own or other farm | |
| Yes | 2.4 (1.1–5.1) |
| No | Reference |
| Contact with poultry at own farm | |
| Yes | 0.5 (0.3–0.8) |
| No | Reference |
| Indirect contact with rats/mice at own farm | |
| Yes | 1.6 (1.0–2.7) |
| No | Reference |
| No. cows on farm in 2008† | 1.0 (1.0–1.0) |
| Farm region | |
| South | 1.9 (1.2–3.1) |
| Other | Reference |
| Automatic milking | |
| Yes | 0.6 (0.4–1.0) |
| No | Reference |
| Birds in stable | |
| Yes | 2.3 (1.2–4.4) |
| No | Reference |
| Contact with cows at other farm | |
| Yes | 1.8 (1.0–3.2) |
| No | Reference |
| Contact with compost | |
| Yes | 0.6 (0.3–0.9) |
| No | Reference |
| Beef cattle on the farm | |
| Yes | 1.9 (1.0–3.7) |
| No | Reference |

*The analysis included the primary participant- and farm-based characteristics associated with positivity (p<0.10 in likelihood ratio test). The number of observations was 708; the number of levels used was 309 (quasi-likelihood under the independence model criterion 695.52). OR, odds ratio.
†Risk increases per cow.

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# Minimal Diversity of Drug-Resistant *Mycobacterium tuberculosis* Strains, South Africa[1]

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Multidrug- (MDR) and extensively drug-resistant tuberculosis (XDR TB) are commonly associated with Beijing strains. However, in KwaZulu-Natal, South Africa, which has among the highest incidence and mortality for MDR and XDR TB, data suggest that non-Beijing strains are driving the epidemic. We conducted a retrospective study to characterize the strain prevalence among drug-susceptible, MDR, and XDR TB cases and determine associations between strain type and survival. Among 297 isolates from 2005–2006, 49 spoligotype patterns were found. Predominant strains were Beijing (ST1) among drug-susceptible isolates (27%), S/Quebec (ST34) in MDR TB (34%) and LAM4/KZN (ST60) in XDR TB (89%). More than 90% of patients were HIV co-infected. MDR TB and XDR TB were independently associated with mortality, but TB strain type was not. We conclude that, although Beijing strain was common among drug-susceptible TB, other strains predominated among MDR TB and XDR TB cases. Drug-resistance was a stronger predictor of survival than strain type.

Drug-resistant tuberculosis (TB) has emerged as a substantial threat to advances in global TB control over the past several decades (*1*). Worldwide, an estimated 630,000 cases of multidrug-resistant (MDR) TB occurred in 2011, and extensively drug-resistant (XDR) TB has now been reported in 84 countries (*2*). MDR TB and XDR TB are each associated with very high mortality rates (*3*), and their transmission—both in community and health care settings—remains an ongoing challenge in resource-limited settings and in countries with high rates of HIV co-infection.

In South Africa, the incidence of MDR TB has increased 5-fold since 2002 (*2*,*4*). MDR TB treatment is now estimated to consume more than half of the budget allocated for TB control in South Africa (*5*). The emergence of XDR TB, and its associated high mortality rates, have further underscored the need for clarifying the factors driving the drug-resistant TB epidemic to better focus control efforts (*3*,*6*,*7*).

Drug-resistant TB is generally considered a human-made phenomenon that occurs when inadequate TB treatment creates selection pressure for the emergence of drug-resistant Mycobacterium tuberculosis subpopulations (acquired resistance) (*1*). Researchers initially believed that the mutations causing drug resistance would exert a "fitness cost," rendering those strains too weak to be transmitted (*8*,*9*). Nonetheless, transmission of drug-resistant TB strains has now been well-documented (*10–13*), and laboratory studies have shown that clinical strains may have minimal fitness costs or even none (*14*). Emerging data suggest that most MDR TB and XDR TB cases in South Africa and worldwide are likely caused by primary transmission of drug-resistant strains (*2*,*15–19*).

Although the *M. tuberculosis* W/Beijing strain family has been described among cases of drug-susceptible, MDR TB, and XDR TB in South Africa, numerous other strain types have also been identified (*20*,*21*). Little is known about the transmissibility and virulence of *M. tuberculosis* strains aside from the W/Beijing strain family (*22*,*23*). In the Eastern Cape and Western Cape Provinces of South Africa, strains from the W/Beijing family have most often been

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associated with transmission of drug-resistant TB (*24*–*27*). At our study site in KwaZulu-Natal Province, however, the LAM4/KZN strain type has predominated among MDR TB and XDR TB cases and has been linked to nosocomial transmission and high mortality rates (*3*,*16*,*17*,*28*,*29*). This strain is a member of the Euro-American strain family and was first described in this region in 1994, evolving into an increasingly resistant phenotype over time (*29*).

The reasons for why the LAM4/KZN strain is prominent in KwaZulu-Natal Province, rather than the Beijing strain, which is seen globally and in other parts of South Africa, is unclear. Moreover, it is unknown whether the higher mortality among patients with MDR TB and XDR TB in KwaZulu-Natal can be explained, in part, by a difference in genotypic prevalence and associated differences in strain virulence (*3*,*6*,*7*,*28*). In this study, we sought to characterize the genotypic diversity of *M. tuberculosis* strains among isolates causing drug-susceptible TB, MDR TB, and XDR TB in KwaZulu-Natal Province, South Africa. We also examined the relationship between *M. tuberculosis* strain, drug resistance, and patient survival.

## Methods

### Study Design and Population

We performed a retrospective study of patients who had received diagnoses of drug-susceptible TB, MDR TB, and XDR TB in Tugela Ferry, KwaZulu-Natal Province, from January 1, 2005, through December 31, 2006. Patients were eligible if their medical records and an *M. tuberculosis* isolate were available for analysis (*30*). The study was approved by the institutional review boards at the University of KwaZulu-Natal, Albert Einstein College of Medicine, and Yale University, and by the KwaZulu-Natal Department of Health.

### Setting

Tugela Ferry is a town situated in a rural district with a population of 200,000 persons. A single, 355-bed government district hospital provides inpatient care. In 2006, the incidence of drug-susceptible TB was 1,100 cases/100,000 population, and MDR TB incidence was 119 per 100,000 persons (*3*). More than 80% of TB case-patients were co-infected with HIV, and the antenatal HIV prevalence was 37%.

Since June 2005, after a large cluster of MDR TB and XDR TB cases were discovered in Tugela Ferry, clinicians there have been encouraged to evaluate all persons with suspected TB by ordering mycobacterial culture and drug-susceptibility testing (DST) in addition to smear microscopy. This practice differed from South African national policy, which recommended culture and DST be requested only when patients were experiencing treatment failure or receiving re-treatment (*31*). Detailed methods regarding sputum collection, microscopy, culture, and DST have been previously described (*28*).

All new TB patients began empiric first-line therapy (administration of isoniazid, rifampin, ethambutol, and pyrazinamide for 2 months, followed by administration of isoniazid and rifampin for 4 months), whereas re-treatment patients began a standard category II regimen (*31*). Second-line therapy for drug-resistant TB was not available at the Tugela Ferry hospital. Patients with confirmed MDR TB or XDR TB were transferred to a referral hospital in Durban for treatment of drug-resistant TB. The average time from sputum collection to transfer was 111 days for XDR TB patients (*4*), during which time patients remained on the inpatient wards receiving first-line TB therapy.

Upon transfer to the TB referral hospital, MDR TB patients received a standardized treatment regimen of kanamycin, ofloxacin, ethionamide, ethambutol, pyrazinamide, and terizidone for at least 4 months, followed by the same regimen without kanamycin for an additional 18 months. XDR TB patients received the same regimen until 2007, when capreomycin and para-aminosalicylic acid became available in South Africa and replaced kanamycin and ofloxacin. Third-line TB drugs and surgical treatment were not routinely used at the time of this study.

### Medical Record Review and Genotyping

Medical records were reviewed for the following patient characteristics: sex, age, HIV history (HIV status, CD4 count, viral load, receipt of antiretroviral therapy), TB history (acid-fast bacilli smear status, presence of extrapulmonary TB, previous treatment episodes), previous hospitalizations, whether patients were referred for second-line TB therapy, and survival. TB isolates underwent spoligotyping using a commercially available kit. Spoligotype patterns were classified according to the 4th International Spoligotyping Database.

### Analysis

We described spoligotype distribution among drug-susceptible TB, MDR TB, and XDR TB isolates by using simple frequencies and proportions overall, and stratified by HIV status. Duplicate isolates from the same patient were included in the study only if they differed in drug resistance pattern or spoligotype. To provide a comprehensive description of the genotypic diversity found, each isolate was reported in the respective drug resistance or spoligotype groups. Thus, the number of isolates exceeds the number of patients in the description of spoligotype distributions.

We tested the association between spoligotype pattern and survival among MDR TB and XDR TB patients by bivariate and multivariable analysis, using product limit estimates and Cox proportional hazards analysis. To account for

patients with multiple isolates of differing drug resistance or spoligotype pattern, we analyzed drug resistance group and spoligotype as time-dependent covariates. When 2 isolates were collected from a single subject on the same day, the bivariate and multivariate analyses were first run by using the less-resistant isolate and then by using the more- resistant isolate for sensitivity analysis. The direction and magnitude of the results did not change regardless of the technique (data not shown). Additionally, to account for missing CD4 counts for multivariable analysis, we performed multiple imputation using a Markov Chain Monte Carlo method as previously described (*30*).

## Results

There were 227 patients who contributed 297 TB isolates for this study. Eighty-six (38%) patients had drug-susceptible TB, 67 (30%) had MDR TB; and 74 (33%) had XDR-TB. The median age was 33–34 years among patients in each drug resistance group (Table 1). More than 90% of patients were HIV co-infected, with a median CD4 count of <100 cells/mm$^3$. The majority of patients had positive acid-fast bacilli smear results, and nearly one-quarter had both extrapulmonary and pulmonary TB disease. Approximately 70% of patients with MDR TB or XDR TB had previously received TB treatment, whereas 34% of patients with drug-susceptible TB had been previously treated. Recent hospitalization was also more common among patients with MDR TB or XDR TB (52% and 59%, respectively), than among those with drug-susceptible TB (21%, p<0.0001).

### Strain Diversity

Among the 297 isolates analyzed, we found 49 different spoligotype patterns (Table 2). The distribution of spoligotypes varied between drug-resistance categories; as drug resistance increased, strain diversity decreased (p<0.0001 for trend) (Figure 1).

Thirty-eight different spoligotype patterns were identified among the 115 drug-susceptible TB isolates (Table 2; Figure 1). W/Beijing strain (ST1) was most common, accounting for 27% (n = 31) of isolates, followed by ST33 (10%; n = 12). The remaining 72 isolates were distributed over 36 unique spoligotype patterns (Table 2; Figure 1).

Three predominant spoligotype patterns were found among the 79 MDR TB isolates (ST34, ST60, and ST53) and accounted for 69% (n = 54) of isolates. The S/Québec family (ST34) was most common (n = 27, 34%), followed by the LAM4/KZN family (ST60, n = 21, 27%) and the T1 family (ST53, n = 6, 8%). The Beijing family (ST1) occurred in 2 (3%) MDR TB isolates. The remaining 23 MDR TB isolates exhibited 13 different spoligotype patterns (ST37, ST42, ST62, ST90, ST92, ST244, ST583, ST766, ST831, ST926, ST1166, ST1547, and ST1750).

The least genotypic diversity was seen among XDR TB isolates: 89% (n = 82) of isolates identified as LAM4/KZN strain (ST60). The T1 strain (ST53) was seen in 4 (4%) isolates. The remaining 6 isolates each had distinct spoligotype patterns (ST33, ST42, ST90, ST136, ST336, and ST1166). None of the XDR TB strains were from the Beijing family.

### Mortality

Overall, 148 (65%) patients died within 1 year of receiving a diagnosis of drug-resistant TB. Risk factors for death have been described previously and included drug resistance group, positive acid-fast bacilli smear, low CD4 count, presence of extrapulmonary disease, and recent

Table 1. Demographic and clinical characteristics of patients with drug-susceptible, MDR TB, and XDR TB, Tugela Ferry, KwaZulu-Natal Province, South Africa 2005–2006*

| Characteristic | Drug-susceptible TB | MDR TB | XDR TB |
|---|---|---|---|
| Total no. | 86 | 67 | 74 |
| Female sex | 31 (36) | 34 (51) | 38 (51) |
| Age, y, median (IQR) | 34 (29–42) | 34 (29–43) | 33 (29–40) |
| Tested for HIV | 68 (85) | 49 (73) | 63 (79) |
| HIV-positive† | 61 (90) | 45 (92) | 63 (100) |
| CD4 available at diagnosis | 33 (54) | 30 (67) | 32 (51) |
| Median cells/mm$^3$ (IQR) | 74 (29–129) | 85.5 (47–217) | 94 (46.5–169) |
| Viral load available at diagnosis | 20 (33) | 11 (24) | 15 (24) |
| Median copies/mL (IQR) | 110,000 (23,000–570,000) | 120,000 (2,000–200,000) | 71,000 (89–530,000) |
| Receiving ARV therapy at diagnosis | 19 (31) | 12 (27) | 18 (29) |
| Sputum smear result available | 80 (93) | 65 (97) | 72 (97) |
| Positive | 45 (56) | 43 (66) | 50 (69) |
| Presence of extrapulmonary TB | 20 (23) | 17 (25) | 22 (30) |
| Previous TB treatment† | | | |
| Any | 29 (34) | 47 (70) | 53 (72) |
| Previous hospitalization† | | | |
| Past 2 y | 18 (21) | 35 (52) | 44 (59) |

*N = 227; values indicate no. (%) unless otherwise noted. MDR TB, multidrug-resistant tuberculosis; XDR TB, extensively drug-resistant TB; IQR, interquartile range; ARV, antiretroviral.
†p<0.05.

Table 2. Spoligotype patterns of *Mycobacterium tuberculosis* isolates from patients in Tugela Ferry, KwaZulu-Natal Province, South Africa, 2005–2006*†‡

| Lineage | Shared type | International family | Octal code | DS TB strains, n = 115 | MDR TB strains, n = 79 | XDR TB strains, n = 92 |
|---|---|---|---|---|---|---|
| **Beijing** | **1** | **Beijing** | **000000000003771** | **31** | **2** | **0** |
| LAM | 4 | LAM3/S | 000000007760771 | 2 | 0 | 0 |
| | 33 | LAM3 | 776177607760771 | 12 | 0 | 1 |
| | 42 | LAM9 | 777777607760771 | 1 | 1 | 1 |
| | **60** | **LAM4** | **777777607760731** | **7** | **21** | **82** |
| | 211 | LAM3 | 776137607760771 | 2 | 0 | 0 |
| | 811 | LAM4 | 777777604060731 | 1 | 0 | 0 |
| | 1321 | LAM1-LAM4 | 677777607760731 | 1 | 0 | 0 |
| | 1624 | LAM3-LAM6 | 776177607560771 | 1 | 0 | 0 |
| | 1750 | LAM4 | 777767607760731 | 0 | 1 | 0 |
| S family | **34** | **S** | **776377777760771** | **4** | **27** | **0** |
| | 466 | S | 776377377760771 | 1 | 0 | 0 |
| | 831 | S | 776367777760771 | 1 | 2 | 0 |
| T family | 37 | T3 | 777737777760771 | 0 | 5 | 0 |
| | 39 | T4-CEU1 | 777777347760471 | 1 | 0 | 0 |
| | 53 | T1 | 777777777760771 | 10 | 6 | 4 |
| | 118 | T2 | 777767777760771 | 1 | 0 | 0 |
| | 205 | T1 | 737777777760771 | 2 | 0 | 0 |
| | 244 | T1 | 777777777760601 | 2 | 1 | 0 |
| | 334 | T1 | 577777777760771 | 1 | 0 | 0 |
| | 358 | T1 | 717777777760771 | 1 | 0 | 0 |
| | 719 | T1 | 776177407760771 | 3 | 0 | 0 |
| | 766 | T1 | 777761007760771 | 0 | 2 | 0 |
| | 879 | T1 | 777767777760671 | 1 | 0 | 0 |
| | 926 | T1 | 773777777760771 | 0 | 5 | 0 |
| | 1166 | T1 | 777377777760771 | 0 | 1 | 1 |
| | 1547 | T3 | 777727777760771 | 0 | 1 | 0 |
| X family | 200 | X3 | 700076777760700 | 1 | 0 | 0 |
| | 336 | X1 | 777776777760731 | 0 | 0 | 1 |
| | 1751 | X3 | 700066777760771 | 1 | 0 | 0 |
| Haarlem | 47 | H1 | 777777774020771 | 2 | 0 | 0 |
| | 50 | H3 | 777777777720771 | 2 | 0 | 0 |
| | 62 | H1-variant1 | 777777774020731 | 1 | 1 | 0 |
| | 75 | H3 | 777767777720771 | 1 | 0 | 0 |
| | 294 | H3 | 577777777720771 | 1 | 0 | 0 |
| Other | 21 | CAS1-Kili | 703377400001771 | 3 | 0 | 0 |
| | 26 | CAS1-Delhi | 703777740003771 | 3 | 0 | 0 |
| | 71 | EAI-undefined | 776337777760771 | 1 | 0 | 0 |
| | 172 | U | 777777777740771 | 1 | 0 | 0 |
| | 374 | U | 777777776000771 | 1 | 0 | 0 |
| | 583 | MANU2 | 777737777763771 | 1 | 1 | 0 |
| | 806 | EAI1-SOM | 757777777413731 | 1 | 0 | 0 |
| | 1092 | CAS | 702777740003771 | 3 | 0 | 0 |

*DS TB, drug-susceptible tuberculosis; MDR TB, multidrug-resistant TB; XDR TB, extensively drug-resistant TB. Boldface indicates the most common strain for each resistance group.
†Does not include 11 isolates with unknown drug-susceptibility test results.
‡See online Technical Appendix (wwwnc.cdc.gov/EID/article/20/3/13-1083-Techapp1.pdf) for all spoligotype patterns in binary format.

hospitalization (*30*). In this study, mortality was additionally found to be associated with TB strain genotype in bivariate analysis: ST60 (KZN strain) and ST34 (Québec) were both associated with increased mortality, whereas ST1 (Beijing strain) was not (Figure 2).

According to multivariate analysis, however, MDR TB and XDR TB remained independently associated with mortality (MDR hazard ratio [HR] 3.37, p<0.0001; and XDR HR 6.75, p<0.0001), but TB strain type did not (Table 3). Low CD4 count, presence of extrapulmonary TB, and recent hospitalization also remained independently associated with mortality.

## Discussion

We examined the genotypic diversity among *M. tuberculosis* strains causing drug-susceptible TB, MDR TB and XDR TB strains from 2005–2006 to better understand the predominance of the LAM4/KZN strain among XDR TB cases in Tugela Ferry, KwaZulu-Natal. We found that a wide variety of TB strains existed among patients with drug-susceptible TB; however, only a subset of strain families were found as the degree of drug resistance increased to MDR TB and XDR TB. The decrease in genetic diversity with increasing drug resistance suggests clonal expansion of MDR TB and XDR TB strains.

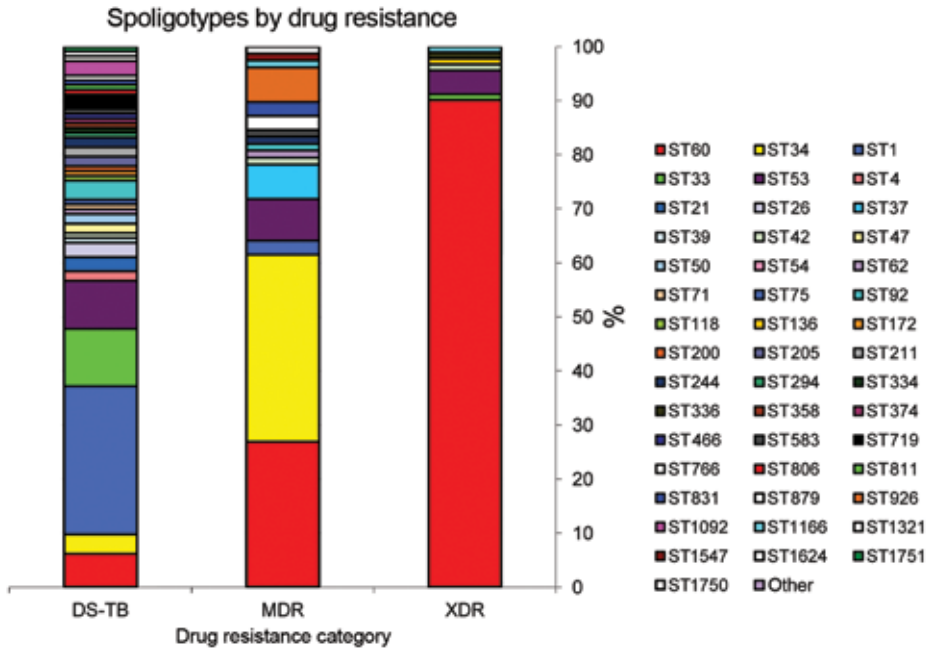


Figure 1. Distribution of spoligotype patterns among drug-susceptible (DS-TB), multidrug-resistant (MDR) tuberculosis and extensively drug-resistant (XDR) cases in Tugela Ferry, KwaZulu-Natal Province, South Africa, 2005–2006. Does not include 11 isolates with unknown drug-susceptibility test results.

Research over the past decade on the drug-resistant TB epidemic in South Africa has uncovered regional differences in the molecular epidemiology of the disease (*25*). Our first report of 53 patients with XDR TB from Tugela Ferry showed that a single strain, the LAM4/KZN strain, accounted for >85% of cases (*28*); subsequent studies have confirmed that the LAM4/KZN strain predominates among drug-resistant isolates throughout KwaZulu-Natal Province (*16*,*25*,*29*). In contrast, studies from South Africa's Western and Eastern Cape Provinces found that 54%–69% of MDR TB and XDR TB isolates belonged to the Beijing family (*25*,*27*). Strains causing drug-resistant TB cases from other provinces varied further; S, T1, and other families accounted for most cases (*20*,*25*). The reasons for these geographic differences remain uncertain. However, the findings from this study allow us to exclude the possibility that the difference exists because the LAM4/KZN strain is endemic among all TB cases and that its predominance among XDR TB cases is simply a reflection of its endemicity. In our study, we found that the LAM4/KZN strain accounted for only 6% of TB cases caused by drug-susceptible isolates and 27% of MDR TB cases. Rather, the same strains that are common among drug-resistant case-patients in other provinces (Beijing, S, T1) are also common among patients with drug-susceptible TB in KwaZulu-Natal.

These data allow us to consider potential causes for the emergence of drug resistance in KwaZulu-Natal at the beginning of the XDR TB epidemic. Drug-resistant TB

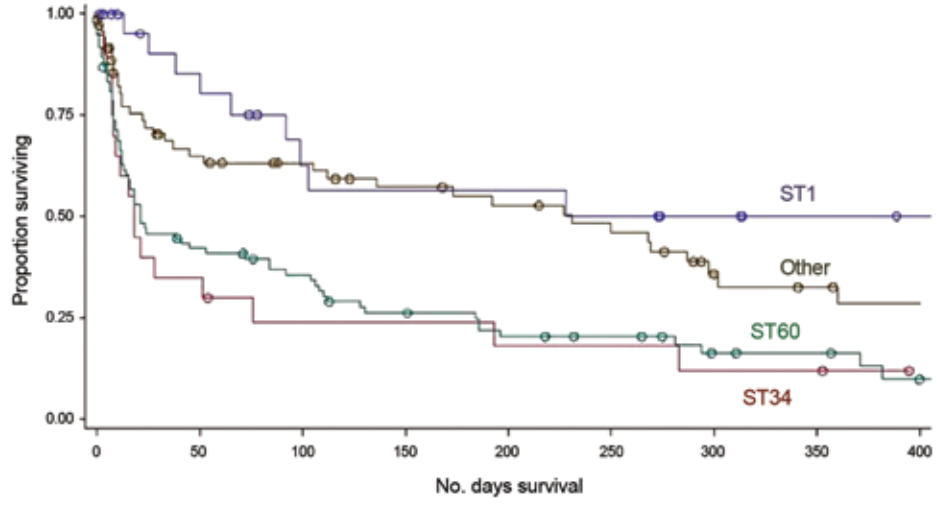


Figure 2. Kaplan-Meier survival distribution, from date of initial sputum collection, stratified by spoligotype (ST1 [Beijing], ST60 [LAM4/KZN], ST34 [S/Quebec] and all others).

Table 3. Association of clinical factors and TB genotype with mortality based on Cox proportional hazards analysis, Tugela Ferry, KwaZulu-Natal Province, South Africa, 2005–2006*†

| Category | Unadjusted hazard ratio | p value | Adjusted hazard ratio | p value |
|---|---|---|---|---|
| Spoligotype (ST) pattern | Ref | | Ref | – |
| ST60 (KZN) | 2.78 | <0.0001 | 0.14 | 0.60 |
| ST34 (Québec) | 2.48 | 0.002 | 0.65 | 0.07 |
| ST1 (Beijing) | 0.66 | 0.26 | 0.72 | 0.42 |
| All other ST types | Ref | | Ref | |
| DST group: drug-susceptible | Ref | | Ref | |
| MDR | 3.37 | <0.0001 | 3.33 | <0.0001 |
| XDR | 5.78 | <0.0001 | 4.68 | <0.0001 |
| Positive sputum smear | | | 1.32 | 0.20 |
| Extrapulmonary TB | | | 1.67 | 0.02 |
| CD4 count | | | | |
| <50 cells/mm$^3$ | | | 2.46 | 0.007 |
| 51–200 cells/mm$^3$ | | | 1.41 | 0.30 |
| >200 cells/mm$^3$ | Ref | | Ref | – |
| Recent hospitalization | | | 2.81 | <0.0001 |

*Ref, referent; KZN, KwaZulu-Natal DST; drug susceptibility testing; MDR, multidrug-resistant, XDR, extensively drug resistant.
†If a patient had differing isolates on the same day, the less resistant of the 2 was used for these analyses. Results did not change when the more resistant isolate was used (data not shown).

occurs either as a result of acquired resistance—selection of resistance in individual patients due to incomplete or improper treatment—or through person-to-person transmission of drug-resistant strains. If acquired resistance were the predominant cause of drug-resistant TB cases, one would expect to find the same TB strains among MDR TB patients as in patients with drug-susceptible TB. By extension, acquired resistance would result in the same strains occurring among XDR TB patients as in MDR TB patients. In this study, however, most strain types prevalent in the drug-susceptible group were absent from the MDR TB strains, and most strains found in the MDR TB group were not found among XDR TB strains. Moreover, a few strains accounted for most MDR TB and XDR TB cases, suggesting clonal expansion. This study builds upon other evidence at the time of data collection (2005–2006), and more recently, which suggests that transmission of drug resistance played a major role in the MDR TB and XDR TB epidemic (*15*–*19*).

It has been hypothesized that certain TB strains have a greater ability than others to spread within a population (*22*,*23*). Numerous studies have suggested that the successful spread of certain Beijing strains may be due to their "hypervirulence," which in part, involves a greater ability to evade host defenses (*23*). A few studies have also examined LAM4/KZN virulence and have shown they show greater adhesion to and invasion of human alveolar cells than other strains (*32*). LAM4/KZN may be more invasive than Beijing isolates while undergoing oxygen deprivation, a condition that mimics the environment in human granulomas (*33*). In addition, data examining the global population structure of *M. tuberculosis* suggest that certain TB lineages may have adapted over time to be more likely to cause disease in, and be transmitted among, specific sympatric human populations from particular geographic settings (*34*). It is unclear whether the geographic differences in the prevalence of drug-resistant strains in South Africa can be explained by such biological differences, or rather, are caused by local outbreaks related to patterns of human congregation and social mixing. Nonetheless, our study highlights the need for further studies to examine the host–pathogen interaction that may contribute to such geographic differences.

Regardless of whether the geographic differences found were caused by biological or social factors, implementing infection control policies and practices in congregate settings is essential. The clonal expansion seen in this study, along with countless other reports of drug-resistant TB transmission worldwide, highlight the major role that transmission plays in this epidemic (*10*–*13*,*19*). Implementation of rigorous infection control programs may curb transmission and avert a large proportion of future secondary cases (*35*). If such programs were implemented in settings such as KwaZulu-Natal, they may change the trajectory of the drug-resistant TB epidemic in a manner similar to what was seen in the United States in the 1990s (*36*). Unfortunately, to date, infection control programs have not been given priority in KwaZulu-Natal, and the incidence of XDR TB remains high (*37*).

Our study also showed that the association between strain type and mortality was attenuated when adjusted for degree of drug resistance, immune suppression, and extent of disease. This finding contrasts with studies that have shown a greater association of W/Beijing strains with disseminated disease and treatment failure (*38*). A recent study from the United States evaluated 4 lineages (East Asian, Euro-American, Indo-Oceanic and East-African Indian) and found an association between strain lineage and clinical site of disease, suggesting differences in pathogenicity and virulence of some strain types (*39*). However, that study did not include patients infected with *M. tuberculosis* from the LAM4/KZN strain family, nor was drug-resistance evaluated as a covariate.

This study is subject to certain limitations. First, the isolates in this study were obtained from patients with culture-positive TB disease for whom spoligotype results were available. Culture-taking practices vary across providers in KwaZulu-Natal and are not routinely obtained for all new patients suspected of having TB. Selection bias may have influenced the strain types found among each drug resistance group in this study. However, the decision to obtain a culture is determined without advance knowledge of strain types, so the differences observed between drug resistance groups are likely to reflect true group differences. Second, the isolates in this study were evaluated by using spoligotyping alone, which was used to assign strain families. More robust methods for defining lineages, such as single nucleotide polymorphism analysis or whole-genome sequencing, may have allowed for more refined assignment of strain families in this study. A recent study, however, directly compared spoligotyping with large sequence polymorphisms and single-nucleotide polymorphisms and found that spoligotyping could be used reliably to classify strain lineages in epidemiologic studies (*40*). In addition, the small sample size in each drug resistance group and known limitations of retrospectively obtained data from chart review may have prevented critical factors independently associated with mortality from being identified. Finally, the study took place at the time of a now, well-characterized, prolonged outbreak of XDR TB in Tugela Ferry. Although the limited genotypic diversity among XDR TB strains could be linked to this outbreak, it would not explain the small number of genotypes seen among isolates causing MDR TB cases at our site, nor the clonality seen among XDR TB isolates from other parts of KwaZulu-Natal (*16*) or in the Eastern Cape and Western Cape Provinces (*25*).

Despite these limitations, this study provides insight into genotypic diversity among drug-susceptible TB, MDR TB, and XDR TB strains in Tugela Ferry, an area with one of the highest rates of HIV and drug-resistant TB worldwide. The decrease in strain diversity with increasing drug resistance provides further evidence that the drug-resistant TB epidemic in KwaZulu-Natal was largely caused by transmission and clonal expansion of predominant strain types. This study also adds to the growing body of literature on strain geographic, clinical, and epidemiologic diversity and provides vital insights for generation of future hypothesis-driven studies of strain virulence and transmissibility. The study results underscore the need for expansion and implementation of sound policy and practices regarding TB/HIV integration and airborne infection control in community and health care facility settings throughout the world.

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# Surveillance for Antimicrobial Drug Resistance in Under-Resourced Countries

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Antimicrobial drug resistance is usually not monitored in under-resourced countries because they lack surveillance networks, laboratory capacity, and appropriate diagnostics. This accelerating problem accounts for substantial number of excess deaths, especially among infants. Infections particularly affected by antimicrobial drug resistance include tuberculosis, malaria, severe acute respiratory infections, and sepsis caused by gram-negative bacteria. Nonetheless, mapping antimicrobial drug resistance is feasible in under-resourced countries, and lessons can be learned from previous successful efforts. Specimen shipping conditions, data standardization, absence of contamination, and adequate diagnostics must be ensured. As a first step toward solving this problem, we propose that a road map be created at the international level to strengthen antimicrobial resistance surveillance in under-resourced countries. This effort should include a research agenda; a map of existing networks and recommendations to unite them; and a communication plan for national, regional, and international organizations and funding agencies.

Antimicrobial drug resistance has become such a global concern that it was the focus of the 2011 World Health Day sponsored by the World Health Organization (WHO). Although antimicrobial drug resistance is well mapped and tightly monitored in some well-resourced countries, such processes do not exist in under-resourced countries. An increasing body of evidence reveals accelerating rates of antimicrobial drug resistance in these countries. Resistance may arise in the absence of any surveillance and threatens the achievement of the Millenium Goals for Development in terms of reduction of maternal and infant deaths (www.un.org/millenniumgoals/). The problem is even more pressing because, in a globalized world, microorganisms and their resistance genes travel faster and farther than ever before, and the pipeline of new drugs is faltering.

Mapping antimicrobial drug resistance in under-resourced countries is urgently needed so that measures can be set up to curb it. Such mapping must rely on efficient surveillance networks, endowed with adequate laboratory capacity, and take into account up-to-date diagnostic techniques. The way forward is to assess the effects of resistance, its clinical effects, and increase in deaths, with the ultimate objective of providing achievable guidelines for surveillance and control.

In this article, we report examples of successful surveillance networks in under-resourced countries and address the framework upon which to deploy reliable and sustainable networks on antimicrobial drug resistance surveillance. This initiative was begun during the Expert Meeting on Diagnosis and Detection of Antimicrobial Resistance in Developing Countries, convened at the Fondation Mérieux Conference Centre "les Pensières," Veyrier-du-Lac, France, October 26–28, 2011.

## What Are the Main Threats?

### Tuberculosis

Resistance *of Mycobacterium tuberculosis* to antimycobacterial drugs is a global concern (Figure). In 2010, an

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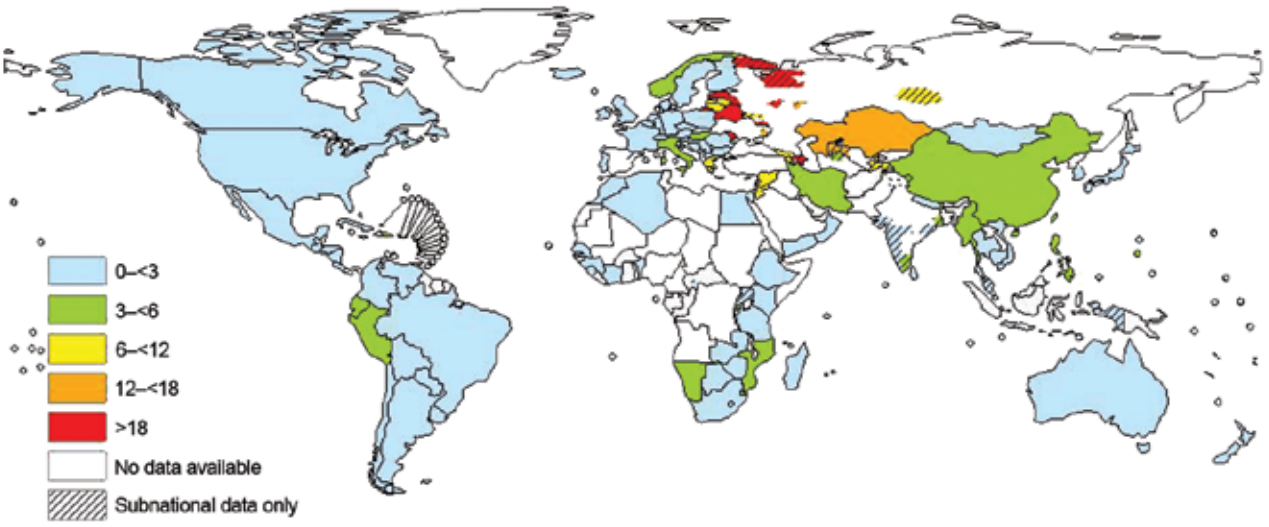


Figure. Proportion of multidrug resistance among strains causing new tuberculosis cases from latest available world data, 1994–2010. Dotted lines on maps represent approximate borders for which there may not yet be full agreement. (Copyright 2011, World Health Organization. All rights reserved.)

estimated 650,000 cases of multidrug-resistant tuberculosis (MDR TB) (i.e., infections with strains resistant to, at minimum, rifampin and isoniazid) occurred worldwide, (*1*). An estimated 10% of cases were extensively drug resistant (XDR) (i.e., MDR strains that are also resistant to second-line drugs). Almost no surveillance system is in place and no data exist on TB resistance in sub-Saharan Africa (apart from South Africa) and Asia.

### Malaria

*Plasmodium falciparum* strains resistant to chloroquine, fansidar, and mefloquine are widespread. Interventions using artemisinin and insecticide-treated bed nets have led to a drop of 40% of malaria cases since 2004 according to WHO. An estimated 750,000 lives were saved in Africa alone (*2*). Those efforts are potentially hindered by the emergence of resistance to artemisinin, which was first reported in 2008 at the Thailand–Cambodia border and subsequently reported in neighboring countries, although not in Africa so far (*3*,*4*). Resistance mechanisms to artemisinin are poorly understood, although mutations in some parasite genes have been partially correlated with resistance (*5*,*6*).

### Severe Acute Respiratory Infections

Severe acute respiratory infections (SARIs) kill an estimated 1.4 million children <5 years of age every year (*7*). The emergence of resistance to neuraminidase inhibitors would potentially have dramatic consequences because they are the first-line response to pandemics caused by highly virulent influenza virus. Resistance of *Streptococcus pneumoniae* to antimicrobial drugs is also a concern. The extent of outpatient penicillin usage correlates with level of resistance (*8*). A prospective surveillance study of 2,184 patients hospitalized with pneumococcal pneumonia in 11 Asian countries in 2008–2009 found that high-level penicillin resistance was rare, that resistance to erythromycin was highly prevalent (72.7%), and that MDR was observed for 59.3% of *S. pneumoniae* isolates (*9*). Of 20,100 cases of invasive pneumococcal diseases identified in South Africa during 2003–2008, a total of 3,708 (18%) were caused by isolates resistant to at least 3 antimicrobial drugs (*10*).

### Gram-negative Bacteria Infections

Gram-negative bacteria resistant to β-lactams are spreading worldwide. CTX-M-15, a heterogeneous and mobile resistance gene first described in 2001 in India, has since been reported all over the world and is transmissible between different species of *Enterobacteriaceae* (*11*). New Delhi metallo-β-lactamase-1 (NDM-1) is a gene that confers resistance to all β-lactams, including carbapenems, which are the only alternative for treating severe infections such as neonatal sepsis caused by MDR strains. First identified in 2008, it is now widespread in *Escherichia coli* and *Klebsiella pneumoniae* isolates from the Indian subcontinent and is found in many countries (*12–18*). Spread of gram-negative resistant bacteria from the hospital to the environment by direct person-to-person contact or through nonsanitized water is a concern in under-resourced countries (*19*). The worldwide increase of the number of travelers, some of whom have diarrhea, is a major cause of

the spread of resistance. A study conducted in Barcelona, Spain, showed that nalidixic acid resistance in enterotoxigenic or enteroaggregative *E. coli* strains isolated from patients returning from India increased from 6% during 1994–1997 to 64% during 2001–2004. Sixty-five percent of strains isolated from patients who had traveled to India were resistant to quinolones (*20*).

#### Methicillin-Resistant *Staphylococcus aureus* Infections

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have become widespread even in under-resourced countries (*21*,*22*). Pakistan and India have reported MRSA percentages of 42%–54.9%, with an increasing trend (*23*).

The above sections describing antimicrobial drug resistance in various diseases is not exhaustive. Careful attention must be given to the potential spread of antimicrobial drug resistance in the drugs used to treat highly prevalent infectious diseases such as typhoid, meningitis, HIV, and hepatitis in under-resourced countries.

## Who Are the Most Vulnerable Populations?

Antimicrobial drug resistance accounts for excess deaths in infants and childbearing women because of poor intrapartum and postnatal infection–control practices (*24*,*25*). In 2005, infections in hospital-born babies were estimated to account for 4% to 56% of all deaths in the neonatal period in some under-resourced countries (*24*). *K. pneumoniae*, *E. coli*, *Pseudomonas* spp., *Acinetobacter* spp., and *S. aureus* were the most frequent causative pathogens of neonatal sepsis; 70% of these isolates would not be eliminated by an empiric regimen of ampicillin and gentamicin. Many infections might be untreatable in resource-constrained environments. Fifty-one percent of *Klebsiella* spp. were extended-spectrum β-lactamase (ESBL) producers, 38% of *S. aureus* strains were methicillin-resistant, and 64% were resistant to co-trimoxazole (*24*). Preliminary data from Kilifi District Hospital (Kenya) also show alarming rates of ESBL positivity: 180 (39%) of 459 *Enterobacteriaceae* clinical isolates from child and adult patients (including 115 isolates of *K. pneumoniae*) collected from August 2010 to August 2012 were ESBL positive (S. Morpeth, unpub. data).

The Division of Women and Child Health at the Aga Khan University Medical College in Karachi, Pakistan, has proposed a model for monitoring the development of neonatal infections and outcomes in southern Asia, on the basis of a cohort of 69,450 births. Resistance rates are constantly increasing. Antimicrobial drug resistance is estimated to result in an additional 96,000 (≈26%, range 16% 37%) deaths each year from neonatal sepsis in southern Asia, highlighting the toll that children pay for drug resistance (Z. Bhutta, unpub. data).

## Promises and Limits of Molecular Diagnostics

The diagnosis of resistance to antimicrobial drugs has so far relied on culture techniques performed in reference centers; these procedures have a long turnaround-time, are technically demanding, and are sometime dangerous. *M. tuberculosis* is difficult to grow, and *Mycobacterium leprae*, *Treponema pallidum,* hepatitis B virus, *Chlamydia pneumoniae*, *Chlamydia trachomatis,* and HIV are impossible to grow. Molecular diagnostic tools offer new promise.

The recent implementation of molecular tools for the diagnosis of *M. tuberculosis* infection and resistance in under-resourced countries illustrates the importance of integrating these tools with traditional diagnostic methods. Mutations within the *M. tuberculosis* genome are associated with resistance. Two PCR-based molecular diagnostic tests were recently introduced with the endorsement of WHO and the financial support of international organizations. GenoType MTB-DRplus (Hain Lifescience, Nehren, Germany) identifies the infectious agent and detects resistance to rifampin and isoniazid in only 2 days with sensitivity and specificity in smear-negative sputum of 94.3% and 96.0%, respectively, compared with drug susceptibility tests (DST) (*26*). A recently released version has sensitivities for the detection of resistance to second-line drugs ranging from 57% to 100% (*27*). Xpert MTB/RIF (CEPHEID, Sunnyvale, CA, USA) diagnoses infection and detects resistance to rifampin with sensitivity and specificity of, respectively, 99.1% and 100%, in 2 hours and in a closed cartridge by using a fully integrated analyzer (*28*).

An immunochromatographic qualitative assay for identifying MRSA, which detects penicillin-binding protein 2a from *S. aureus* isolates (not on specimens), is sold by Alere Inc. (Waltham, MA, USA). CEPHEID produces 2 GeneXpert cartridges to detect MRSA and vancomycin-resistant enterococci on specimens and is currently developing a cartridge for simultaneous detection of 3 major β-lactamase genes (KPC 1–11, NDM 1–6, and VIM 1–32) in rectal swab specimens. Resistance to antiretroviral drugs is usually determined by gene sequencing. None of these assays are extensively used in under-resourced countries.

However, as promising as these diagnostics are, their use would help identify resistance for a limited number of microorganisms. The multiplicity of resistance genes, low inoculum size, variations in permeability to antibacterial drugs, and the activity of efflux pumps are factors influencing antibacterial drug sensitivity. Development of new assays will require research efforts. A novel approach, based on RNA detection of a set of bacterial transcripts after a brief period of antibacterial pulse therapy, may enable rapid differentiation of susceptible and resistant organisms directly from specimens (*29*). Nonetheless, molecular diagnostic tests should not replace culture-based DST in central reference laboratories that are responsible

for updating treatment algorithms and identifying new resistance mechanisms.

## Mapping Antimicrobial Drug Resistance

### Resistance Surveys in Africa and Southeast Asia

A surveillance network, coordinated by the Malaria Research Training Centre (University of Bamako, Bamako, Mali), has taken advantage of rapid molecular-based tests that have been adapted for field use. It relies on health care workers, who have limited clinical training. After a short training course, they are able to collect blood samples from finger pricks and spot them onto filter paper strips that are sent to regional sites that detect PfCRT 76T, the key mutation causing chloroquine resistance. Data are centralized to establish a comprehensive map of resistance. Overall, results showed high prevalence rates (84.5%; range 60.9%–95.1%) across the sites (*30*). Another survey in the district of Kidal identified the mutation in 80% of cases and showed that no isolates carried the dhfr/dhps quintuple mutant (a genetic determinant of pyrimethamine resistance) (*31*). On the basis of these results, chloroquine was replaced by sulfadoxine-pyrimethamine for malaria treatment. Key factors that contributed to the success of this health information system included molecular methods and the reliability of the data-sharing system. Internet access through satellite connection, which became possible a few years ago, will allow more timely data centralization.

A project in 8 West African countries, coordinated by Institut Pasteur and the International Union Against Tuberculosis and Lung Diseases and funded by the Organization of the Petroleum Exporting Countries Fund for International Development, plans to implement GenoType MTBDRplus (Hain Lifescience) on samples positive for *M. tuberculosis* by miscroscopy and Xpert MTB/RIF (CEPHEID) on samples from persons who experienced treatment relapse or failure. These methods will be used in reference centers, together with methods already in use. Another successful example is the South African National TB Drug Resistance Survey (www.mrc.ac.za/operationaltb/DRSUpdate.pdf), which started in June 2012 and plans to enroll 170,000 patients with suspected TB; culture DST will be performed by a sentinel reference laboratory. The survey is supported by several national and international funding agencies. Similarly, the Asian Network for Surveillance of Resistant Pathogens implemented an extensive survey of antimicrobial drug resistance in isolates causing pneumococcal infections in 11 Southeast Asian countries (*9*).

### Role of Health and Demographic Surveillance Sites (HDSS)

Grundmann et al. have published an inventory of preexisting regional surveillance programs in the 6 WHO regions (*32*). The authors suggest that HDSS could serve as focal points for training and dissemination of laboratory and surveillance competencies. HDSS have been established in 39 countries in Asia, Africa, and Oceania as part of the International Network for the Demographic Evaluation of Populations and Their Health in Under-Resourced Countries initiative (INDEPTH; www.indepth-network.org/). HDSS monitor >2 million persons at the household level on a regular basis, have good management capacities, and, as such, create an opportunity for monitoring antimicrobial drug use at the consumer level. A study among children <5 years of age in Kilombero (Tanzania) shows that mapping the patterns of antimicrobial drug use and monitoring resistance is possible based on HDSS, even in a region with only 1 doctor for a population of ≈1 million persons (*33*). In this region, maps, which included houses, hospitals, drug shops, villages, health centers, and the level of antimicrobial drug use revealed that the poorer the family, the less access they had to antimicrobial drugs for their children. On the basis of this map, antimalarial drugs and antibacterial drugs were distributed to the population.

There is considerable potential for use of sentinel population-based studies with monitoring of febrile illnesses (*34*) or routine or diarrheal diseases. Blood cultures and stool specimens can be used to evaluate the prevalence of antimicrobial drug resistance in urban and rural settings (*35*).

### Using Existing Databases

The above-mentioned survey conducted in Karachi emphasizes the need to systematically assess available information from databases such as a hospital's mainframe admissions database, the neonatal intensive care unit admission database, and the microbiology laboratory database to evaluate potential links between antimicrobial drug resistance and major infectious diseases. However, such modeling exercises need to be complemented with prospective surveillance studies.

When measuring antimicrobial drug resistance in under-resourced countries, researchers should consider the epidemiology of the infections and what the denominator is for the surveillance. Too often, published antimicrobial drug resistance data come from hospitals that only wealthy patients can afford to attend, which biases the data.

## Addressing Basic Problems

### Insufficient Laboratory Capacity

Lessons learned from antimicrobial drug resistance surveillance highlight the need to endow surveillance networks in under-resourced countries with laboratory capacity in a sustainable manner—infrastructure and human resources are required to obtain reliable data that can inform both clinicians and policy makers. Common problems

should be addressed first: the death of bacteria during shipping, lack of standardization and guidelines for preparing culture media, high rates of culture contamination, and the lack of availability of diagnostic tests matching the most common infectious agents in circulation in a given area. A survey within the Network for Surveillance of Pneumococcal Disease in the East African Region shows, for example, that an average of 17% of isolates died during shipping (4%–7% from Kenya and nearby Tanzania and 20%–60% from sites further away) and that a frequent cause of this wastage was the shortage of dry ice to keep the isolates frozen (*36*). Other common problems include the lack of harmonization of methods of culture used to grow bacteria, frequent unavailability of required laboratory consumables due to lack of resources and supply-chain problems, and inaccurate training and poor motivation of laboratory technicians and clinicians.

Training, feedback, and the introduction of a few simple hygienic measures implemented over the past 3 years in the pediatric service at Kilifi District Hospital have resulted in a reduction of the rates of contamination in blood culture samples from children from 19% in January 2009 to 5% in July 2011 (S. Morpeth, unpub. data). Good coordination between clinical and laboratory staff was crucial for this achievement, but the backing of the hospital senior clinical management staff was also necessary. However, the effort required to achieve similar results in standard district hospitals will obviously be more intense than it was at Kilifi District Hospital, which is part of a major research program. Clinical demand and political will are critical for the development of adequate laboratory services.

Existing infrastructures in under-resourced countries encompass ingredients for good surveillance of antimicrobial drug resistance, but the bottleneck is laboratory capacity. This capacity needs to be built into all levels of the health care system. Planning should take into account the following: tools to be used for diagnosis, sustainable funding, sharing of standard operating procedures, data management, a central database, and resource centers. In addition, logistical and human issues should be considered: for example, common procurement strategies, training of technicians, communication to raise awareness of antimicrobial resistance, feedback of results to local clinicians and central policy makers, and identification of the leaders who will support the development of surveillance networks.

### Standardization of Resistance Data

Standardization is critical for comparison and globalization of surveillance. The Clinical and Laboratory Standards Institute in the United States and the European Union Commission do not agree on the values of MICs or disk diffusion zone size break points for antibacterial drugs, and the introduction of molecular techniques may further complicate comparisons. Setting up a resistance index that would make the data comparable across different regions would be a valuable research objective.

## Building on Successful Experiences

### European Antimicrobial Resistance Surveillance Scheme (EARSS)

Drawing on lessons learned from the development of EARSS, a surveillance system that monitors antimicrobial drug resistance in Europe and other networks, we strongly recommend that countries start with good quality data from a limited number of sites. Similarities between the situation in Europe when EARSS started its surveillance activities and the situation in Africa can be pointed out. EARSS has been gradually scaled up from a starting point in 1998 when only 2 bacteria were monitored in 78 laboratories in 7 countries. In 2011, EARSS encompassed 977 laboratories and 1,577 hospitals in 33 countries covering a population of >700 million citizens (*37*). This experience could guide the development of surveillance in under-resourced countries.

### Using Existing Networks

Surveillance networks developed for specific purposes could serve as the basis for monitoring other infectious agents and their resistance to antimicrobial drugs. For example, initiatives are ongoing within the Organisation de Coordination pour la Lutte contre les Endémies en Afrique Centrale to foster the awareness of antimicrobial drug resistance in Africa (B. Gicquel, unpub. data). Similarly, blood culture facilities installed as part of malaria vaccine trials provide a background infrastructure that could be built on (*32*).

## Perspectives and Recommendations

The Table shows recommendations from the group that met at the Expert Meeting on Diagnosis and Detection of Antimicrobial Resistance in Under-Resourced Countries. A roadmap for the development of effective surveillance networks, endowed with good laboratory capacities, is urgently needed. Surveillance should also be integrated with other public health measures aimed at curbing the spread of pathogens, such as vaccination campaigns. Conjugate vaccines, for example, have been shown to reduce the antibacterial drug resistance of *S. pneumoniae* strains (*38*). Surveillance should thus be part of a global and coordinated strategy for control of antimicrobial drug resistance in under-resourced countries. The roadmap should follow a gradual process, starting with a limited number of pathogens, because attempting to address everything everywhere could kill the project. The meeting participants agreed on the necessity of establishing the roadmap with 3 key items:

Table. Recommendations for efficient antimicrobial drug–resistance surveillance in under-resourced countries

| Recommendation |
|---|
| Laboratory improvement |
| Address both patient management and surveillance needs |
| Build sustainable capacity (infrastructure, equipment, human resources) |
| Provide good coordination between clinics and laboratory |
| Standardize procedures |
| Identify appropriate diagnostic tests for antimicrobial drug resistance (e.g., molecular tests for uncultivable or slow-growing bacteria or for organisms in which resistance is linked to a single gene |
| Logistical needs |
| Avoid shortage of reagents; address both resources and supply chain |
| Ensure appropriate specimen collection and transport to the laboratory |
| Political will |
| Backed by hospital management |
| Endorsed by policy makers |
| Standardized antimicrobial drug resistance results: resistance index |
| Leverage successful experiences |
| Integrate drug resistance surveillance to other public health measures aimed at curbing the spread of pathogens |
| Start small; increase gradually |
| Take advantage of existing networks targeting specific diseases (HIV, malaria, tuberculosis) |

a research agenda, a map of existing initiatives and networks, and a communication plan aimed at international organizations and funding bodies.

### Research Agenda

The research agenda should focus on developing appropriate technology for antimicrobial drug resistance detection, leveraging novel technologies that have emerged recently. The group will review these technologies and build a proposal for funding agencies.

### Mapping Existing Initiatives

Mapping existing initiatives will be a key component of the roadmap. Recently, several such initiatives have been launched. In 2011, WHO emphasized the need to strengthen efforts recommended in its 2001 Global Strategy for Containment of Antimicrobial Resistance, for example through the strategic action plan on antimicrobial drug resistance of the WHO European region. The WHO Patient Safety Program published in 2012 The Evolving Threat of Antimicrobial Resistance: Options for Action (www.who.int/patientsafety/implementation/amr/publication/en/), which lists some networks involved in antimicrobial drug resistance surveillance activities, including those in Africa, Southeast Asia, and Latin America. In 1911, the European Union (EU) proposed an Action Plan against the rising threat of antimicrobial drug resistance. Several EU initiatives should support activities in under-resourced countries: the Joint Programming Initiative on Antimicrobial Resistance (http://www.jpiamr.eu/) gathered 17 EU countries and proposes to set 10 million €/year for 5 years for research; the Innovative Medicine Initiative, a public–private partnership between EU and the pharmaceutical industry association; the European Federation of Pharmaceutical Industries and Associations, which edits regular calls for proposals within its program Combating Antibiotic Resistance: New Drugs for Bad Bugs,the next EU program for research and innovation (Horizon 2020); and the European and Under-resourced Countries Clinical Trials Partnership. EU and the United States support the Transatlantic Task Force on Antimicrobial Resistance, which recently reviewed its recommendations to keep antimicrobial agents effective (www.ecdc.europa.eu/en/activities/diseaseprogrammes/TATFAR/Documents/210911_TATFAR_Report.pdf). Most of these initiatives are targeting the well-resourced world. There is an urgent need to advocate for consideration of specificities and constraints of preventing antimicrobial drug resistance, especially surveillance, in low- and middle-income countries and to implement existing global plans, with substantial and sustainable funding provided to these countries.

### Detailed Communication Plan

Advocacy for surveillance for antimicrobial drug resistance, based on a detailed communication plan, should also target health authorities in the concerned regions. The Health Ministers of WHO South-East Region member states agreed in 2012 on 18 recommendations at national and regional levels for preventing and containing antimicrobial drug resistance, including increased capacity for surveillance (http://209.61.208.233/LinkFiles/WHD-11_antimicrobial drug resistance_Jaipur_declaration_.pdf). This call for action should be reproduced in other WHO regions, especially in the region for Africa.

To build this 3-item roadmap, the proposed initiative should join efforts with programs like the Global Antibiotic Resistance Partnership, which develops actionable policy proposals on antibiotic resistance for low- and middle-income countries through a network with members in India, Kenya, South Africa, Vietnam, Mozambique, and Tanzania (www.cddep.org/projects/global_antibiotic_resistance_partnership); GABRIEL (Global Approach for Biological Research on Infectious Epidemics in

Low-Income Countries), coordinated by Fondation Mérieux, with laboratories in 17 under-resourced countries; or the International Network of Pasteur Institutes, found in 32 countries.


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# Drought and Epidemic Typhus, Central Mexico, 1655–1918

**Jordan N. Burns, Rodolfo Acuna-Soto, and David W. Stahle**

Epidemic typhus is an infectious disease caused by the bacterium *Rickettsia prowazekii* and transmitted by body lice (*Pediculus humanus corporis*). This disease occurs where conditions are crowded and unsanitary. This disease accompanied war, famine, and poverty for centuries. Historical and proxy climate data indicate that drought was a major factor in the development of typhus epidemics in Mexico during 1655–1918. Evidence was found for 22 large typhus epidemics in central Mexico, and tree-ring chronologies were used to reconstruct moisture levels over central Mexico for the past 500 years. Below-average tree growth, reconstructed drought, and low crop yields occurred during 19 of these 22 typhus epidemics. Historical documents describe how drought created large numbers of environmental refugees that fled the famine-stricken countryside for food relief in towns. These refugees often ended up in improvised shelters in which crowding encouraged conditions necessary for spread of typhus.

Epidemic typhus is a serious infectious disease caused by the obligate, intracellular, gram-negative bacterium *Rickettsia prowazekii* and transmitted by body lice (*Pediculus humanus corporis*). This disease is recognized for its high mortality rate throughout human history, particularly before modern sanitary practices and the availability of antimicrobial drugs (*1*). Typhus spreads where conditions are crowded and unsanitary. Historical epidemics often followed war, climate extremes, famine, and social upheaval. Zinsser (*2*) noted that throughout history, epidemic typhus might have claimed more human lives during war than combat. Epidemic typhus could reemerge as a serious infectious disease in areas of the world where social strife and underdeveloped public health programs persist (*3*).

Typhus was first recorded in Mexico in 1655, and the most recent major epidemic began in 1915 during the Mexican revolution. Mexico's rich historical record of epidemic disease is documented in archives of demographic data that include census records, health records, death certificates, and accounts of physicians. Mexico City and the high, densely populated valleys of central Mexico were particularly susceptible to smallpox, cholera, and typhus epidemics because of crowding and poor sanitation (*4*). Numerous epidemics, some identified as typhus, occurred during the colonial and early modern eras. We have compiled a record of 22 typhus epidemics in Mexico during 1655–1918. We compared the timing of these typhus epidemics with tree-ring reconstructions of growing-season moisture conditions to assess the relationship between climate and typhus during this period.

## Background

The historical and geographic origins of typhus are disputed. Despite early evidence for typhus in Europe, it is unclear whether typhus was imported from Europe to the New World during colonization or vice versa (*3*). During the 16th century, typhus fever was gradually distinguished from diseases with similar clinical manifestations as physicians learned to recognize typhus by its sudden onset and characteristic rash (*5*). Epidemic typhus was not precisely distinguished from typhus fever until 1836 (*6*). Tabardillo, or Mexican typhus fever, was considered a disease separate from epidemic typhus until Dr. Howard Ricketts and others in 1910 proved that the 2 diseases were identical (*5*).

Epidemic typhus remains a threat in the rural highlands of South America, Africa, and Asia. Areas of Russia, Burundi, Algeria, and Andean Peru have all experienced typhus outbreaks in the past 20 years and are currently susceptible to outbreaks because of a high incidence of body lice, homelessness, or a large population of typhus survivors who are at risk for Brill-Zinsser disease (*6–9*). The risk for epidemic typhus has not been eliminated from more industrialized regions because body lice infestation still occurs in homeless populations in the United States, Europe, and the Netherlands (*10*).

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Body lice infestation and typhus posed public health problems in Mexico until fairly recently. Cases of louse-borne typhus occurred mainly in cold months and in rural communities. From the late 19th century until 1963, the annual mortality rate of epidemic typhus in the rural state of Mexico decreased steadily from 52.4 to 0.1 cases/100,000 persons, and by 1979 no cases had been reported for 10 years. At the beginning of a 1980s program combating lice infestation, the rate of infestation with *P. humanus corporis* lice in the indigenous population of the state of Mexico was 100%. This rate decreased to 5%–12% by 1999. A 2002 study sampled the seroprevalence of typhus antibodies in ≈400 persons from communities in the state of Mexico. The prevalence of typhus antibodies in persons >65 years of age was 48%, and 6 case-patients who had particularly high levels of antibodies indicated possible Brill-Zinsser disease. Typhus survivors in Mexico are at risk for relapsing typhus fever and are potential sources for typhus outbreaks. An outbreak in Atlacomulco in 1967 was traced to a 76-year-old man with Brill-Zinsser disease (*11*).

## Data Sources and Methods

A total of 22 typhus epidemics during 1655–1918 were identified from historical records of disease in Mexico. Historical documentation of typhus epidemics includes almanacs; diaries; personal accounts; and medical and death records from hospitals, physicians, cemeteries, and municipalities. The terms typhus, symptomatic typhus, and tabardillo used in historical references were taken to mean epidemic typhus. When monthly mortality rate data were available, the onset of an epidemic was identified by an increase in the number of deaths from tens to hundreds over a 2-month period and by multiple historical references to typhus cases. Sources that indicated urgency; desperation; and a widespread need for supplies, medical care, hospitals, and cemeteries help distinguish normal typhus incidence from a typhus epidemic for which detailed mortality rate data were not available. A detailed summary of historical sources, including references, quotations, and a description for each epidemic, has been reported (J.N. Burns, unpub. data, http://cavern.uark.edu/misc/dendro/2013-Burns-Typhus.pdf).

The 22 identified typhus epidemics were compared with tree-ring–reconstructed soil moisture estimates for central Mexico by using 2 methods (Figure 1). The Palmer Drought Severity Index (PDSI) is used to represent the soil moisture balance and is computed from precipitation and temperature measurements incorporated into a numerical 2-layer soil moisture model (*12,15*). Station-based observations of monthly temperature and precipitation were mapped on a 0.5° latitude/longitude grid and then used to calculate monthly PDSI values for each grid point (R.R. Heim, Jr., National Climatic Data Center, Ashville, NC, USA) by using climatically aided interpolation techniques (*16*). A tree-ring reconstructed PDSI value for central Mexico is shown in Figure 1, panel A and a reconstructed PDSI value for east-central Mexico is shown in Figure 1, panel B. Years with mild, moderate, severe, and extreme droughts are indicated by PDSI values of -1, -2, -3, and –4, respectively (J.N. Burns, unpub. data). The corresponding wet conditions are indicated by PDSI values of +1, +2, +3, and +4. The years or periods in which typhus epidemics occurred are specified on each time series.

The reconstruction for central Mexico (Figure 1, panel A) was based on the gridded reconstructions of PDSI values for summers of the past millennium, which were compiled from >1,400 tree-ring chronologies developed across North America, including a few from northern and central Mexico (*15*). We extracted the gridded PDSI value reconstructions for central Mexico (78 grid points) and averaged the yearly estimates of each grid point into a single 500-year time series of summer PDSI value measurements for that region (summer PDSI values = June, July, August averaged PDSI values). Selected grid locations and reconstructed PDSI values for central Mexico have been reported (Burns JN, unpub. data). This time series approximates a history of drought and wetness over central Mexico.

A second reconstruction of PDSI values was made for east-central Mexico by using the earlywood width chronology derived from ancient Douglas fir trees at Cuauhtemoc la Fragua, Puebla (Figure 1, panel B). The Cuauhtemoc collection includes 205 radii from 85 different trees and fallen logs. Each tree ring was exactly dated by using standard dendrochronologic methods described by Douglass (*17*), and earlywood and latewood growth increments were measured by using a stage micrometer. The resultant earlywood width chronology represents a record of drought and wetness in east-central Mexico during 1474–2001. The Cuauhtemoc la Fragua chronology might be the most climate-sensitive long chronology developed for central Mexico and it has been used to study the role of climatic extremes in famine, disease, and social upheaval throughout Mexican history (*14,18*).

The Cuauhtemoc earlywood chronology was used to reconstruct June–July PDSI values for east-central Mexico (i.e., the Sierra Madre Oriental and the Valley of Mexico). The reconstruction was calibrated with instrumental June–July PDSI values for east-central Mexico by using bivariate regression for 1975–2001 ($R^2_{adj} = 0.54$, based on an average of 22 grid points). This June–July PDSI value reconstruction verifies only weakly when compared with independent instrumental PDSI values for 1950–1974 ($r = 0.3$), and the reduction of error and coefficient of efficiency statistics are barely positive (0.003 and 0.003, respectively) (*19*). These marginal verification statistics suggest that the area of the June–July PDSI signal in east-central Mexico recorded by

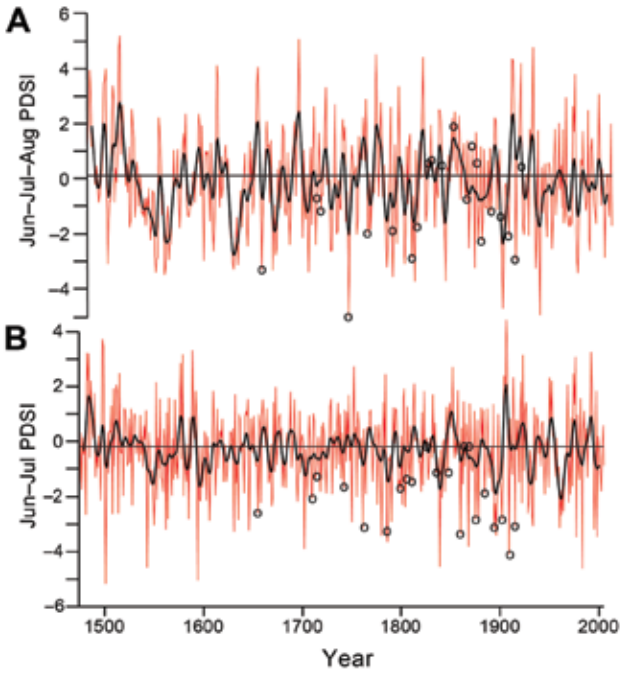


Figure 1. A) Time series of summer Palmer Drought Severity Index (PDSI) averaged for 78 grid points in central Mexico, 1665–1918. Data were obtained from Cook et al. (*12,13*) and Therrell et al. (*14*). B) Time series of June–July PDSI reconstructed from the Cuahtemoc la Fragua tree-ring chronology in east-central Mexico by using an average of 22 grid locations from the monthly PDSI dataset of R.R. Heim, Jr. (National Climatic Data Center, Ashville, NC, USA). Circles indicate typhus epidemics. Red lines indicate high-frequency yearly variability of moisture reconstruction. Black lines indicate smoothed lower-frequency representation of this variability. Horizontal lines indicate average PDSI for period.

the single tree-ring chronology at Cuauhtemoc la Fragua has varied in size during the instrumental era. Nevertheless, the Cuauhtemoc la Fragua Douglas fir chronology has a strong moisture signal for the past 500 years in spite of the weak verification statistics (*14,18*).

The statistical significance of the relationship between drought and epidemic typhus in Mexico during 1655–1918 was assessed by using superposed epoch analysis (*20*). Reconstructed PDSI values were sorted and averaged for years before, during, and after the 22 historical typhus epidemics. The mean PDSI value was computed for the typhus event years or periods and was then tested against the mean of all other years in the reconstructed time series for central (Figure 2, panel A) and east-central Mexico (Figure 2, panel B). The means for each of 6 years before and after each epidemic was also tested against the mean of all other years to examine any cumulative or lag effects of drought on the typhus epidemics.

The reconstructed summer PDSI values for all epidemic years or periods were averaged at each of the 11,396 continent-wide grid points, and these averages were mapped to represent the spatial anomaly of growing season moisture during the catastrophes; an updated version (*13*) of the reconstructions of Cook et al. (*12*) was used. The reconstructed PDSI values for all typhus epidemic years are mapped in Figure 3, panel A, and the PSDI values for the 15 driest epidemic years are mapped in Figure 3, panel B to highlight the area of Mexico most afflicted by drought conditions during these epidemics.

## Historical Typhus Epidemics

Historical citations from Mexico during 1655–1918 leave no doubt that drought and famine were associated with some serious epidemics of typhus. In fact, drought, famine, and/or crop failure was reported by historical sources during 15 of the 22 periods of epidemic typhus and was described as markedly severe or widespread during the epidemics of 1714, 1785, 1861, 1875, 1884, 1909, and 1915 (J.N. Burns, unpub. data). The tone used in historical references concerning these years suggests a state of national or regional emergency, whereas references from less severe epidemics suggest that the outbreaks were localized crises. Drought and a severe early frost coincided during 1785, sharply reducing harvests and causing a famine so widespread that 1785 became "El Año del Hambre" ("the year of hunger") in Mexican history (*18,23*). This year of starvation coincided with the typhus epidemic of 1785. Again, from 1909 through 1911, epidemic typhus coincided with severe frost and a corn crop failure so massive that corn import taxes were eliminated. Concise descriptions of each of the 22 epidemics were synthesized from dozens of historical citations from Mexico and were compiled (J.N. Burns, unpub. data).

Information from historical sources about past climate and typhus epidemics is variable because reports come from many authors and they do not describe events consistently, especially environmental conditions. A more consistent and independent proxy for moisture variability was needed to test the possible role of climate in past typhus outbreaks. We used tree rings because they can be exactly dated and are well correlated with available soil moisture data.

## Tree Ring–Reconstructed Drought

When the 22 typhus epidemics were compared with tree-ring reconstructions of summer PDSI values, we noted below average tree growth and reconstructed drought during the onset year of most typhus epidemics (15 of 22 typhus periods in central Mexico (Figure 1, panel A). Many of these epidemics occurred during years when reconstructed moisture for central Mexico was far below average (Figure 1, panel A). The time series for east-central Mexico indicates that 19 of the 22 epidemics occurred during drought and that only 3 typhus events occurred

during years with near-normal reconstructed moisture estimates (Figure 1, panel B).

The superposed epoch analyses indicate that the reconstructed mean PDSI values for all 22 epidemic periods in central Mexico was significantly below the average of all remaining years (mean –1.16; $p<0.01$) (Figure 2, panel A). This drought pattern was especially pronounced in the reconstructed PDSI values for east-central Mexico during the 22 events (mean –1.68; $p<0.001$) (Figure 2, panel B). These results suggest that drought may have been a major factor in the spread of typhus in colonial and early modern Mexico (Figure 2, panel A).

The continent-wide reconstructed PDSI values mapped in Figure 3, panel A, indicate mild drought conditions over most of Mexico during the 22 periods in which typhus epidemics occurred. When the 7 epidemic years with near-normal to above-average moisture reconstructions are omitted from the mapping of reconstructed PDSI values, drought for the remaining 15 epidemic years appears much more intense, particularly over east-central Mexico (Figure 3, panel B). This finding suggests that the most severe drought during those 15 typhus epidemics might have been located in east-central Mexico, where most of the 22 typhus epidemics were concentrated, according to historical documentation (J.N. Burns, unpub. data).

## Discussion

The observed relationship between drought and typhus epidemics in colonial and modern Mexico is curious because drought has not been specifically considered as a risk factor for typhus. Nevertheless, drought, much like war and natural disaster, has caused famine in poor agricultural regions and can promote migration of impoverished refugees from the countryside into city centers in search of food and relief (*24,25*). An influx of starving persons without money, jobs, or shelter into urban areas in Mexico might have amplified the crowded and unsanitary conditions necessary for historical outbreaks of epidemic typhus.

There is some support for this environmental refugee theory in historical documents from Mexico, in which drought-induced famines and influxes of poor persons into cities were sometimes recorded. For example, a wealthy man described the city of Guanajuato during the typhus epidemic of 1714, when crowds of poor persons gathered in the street to beg for food: "Walking on the streets it was common to find people reduced almost to their bare skeletons walking in bands. With almost no strength they kneeled on front of you and said in a very faint voice 'For God's love, we are dying of starvation, help us in our necessity, you powerful man.' It was painful to see the poor and sick who, because of their great necessity, came out to the streets with all their bodies shaking, surely a bad thing for their disease" (J.N. Burns, unpub. data).

Another account described the typhus epidemic of 1785, which coincided with the year of hunger in city of Queretaro: "The epidemic competes with famine for the number of deaths. In the mountains, hills, streets, was a theater of the saddest spectacle, long caravans of miserable people walked in all directions asking, for God's love, a piece of bread. Very often some of them fell and died shortly after." During this time, according to Cooper (*4*), "concurrently the region suffered 'a series of natural calamities which completely destroyed the grain crop and reduced large sections of the population to a state of extreme poverty and famine. That winter [of 1785] and the spring of 1786 saw thousands of desperate farmers and workmen roaming the countryside, swarming into the towns in search of food, and dying of starvation and disease'" (J.N. Burns, unpub. data).

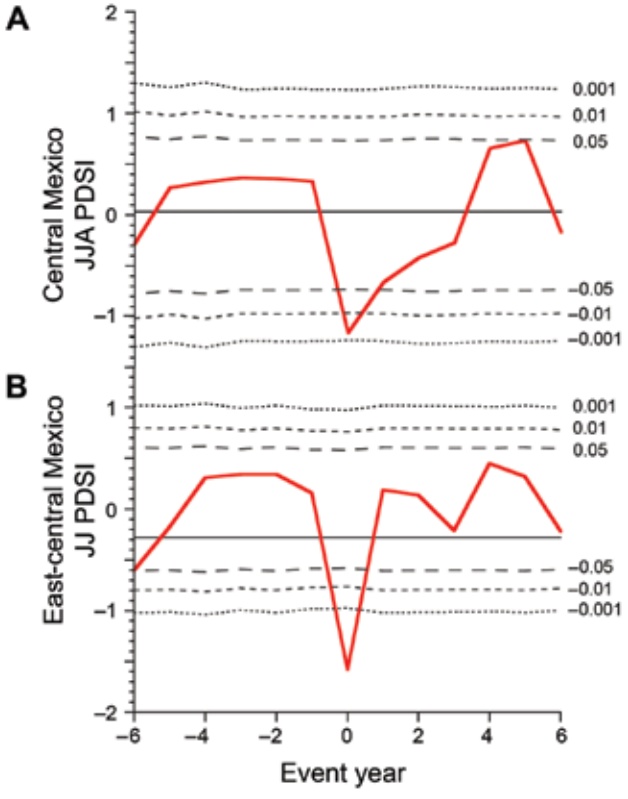


Figure 2. A) Superposed epoch analysis (*20*) of summer Palmer Drought Severity Index (PDSI) for central Mexico averaged for the 22 periods that had typhus epidemics (1655, 1710–1712, 1714, 1742, 1761–1762, 1785–1787, 1799–1802, 1805–1806, 1811–1812, 1821–1823, 1825–1828, 1835–1838, 1847–1848, 1861–1864, 1865–1868, 1870–1873, 1875–1877, 1884–1886, 1894–1895, 1902–1903, 1909–1911, and 1915–1918). Horizontal line indicates PDSI = 0. JJ, June–July; JJA, June–July–August. B) Superimposed epoch analysis of June–July PDSI for east-central Mexico also averaged for the 22 periods that had typhus epidemics. Superimposed epoch analyses were performed by using the Dendrochronology Program Library (*21,22*). Horizontal line indicates mean PDSI.

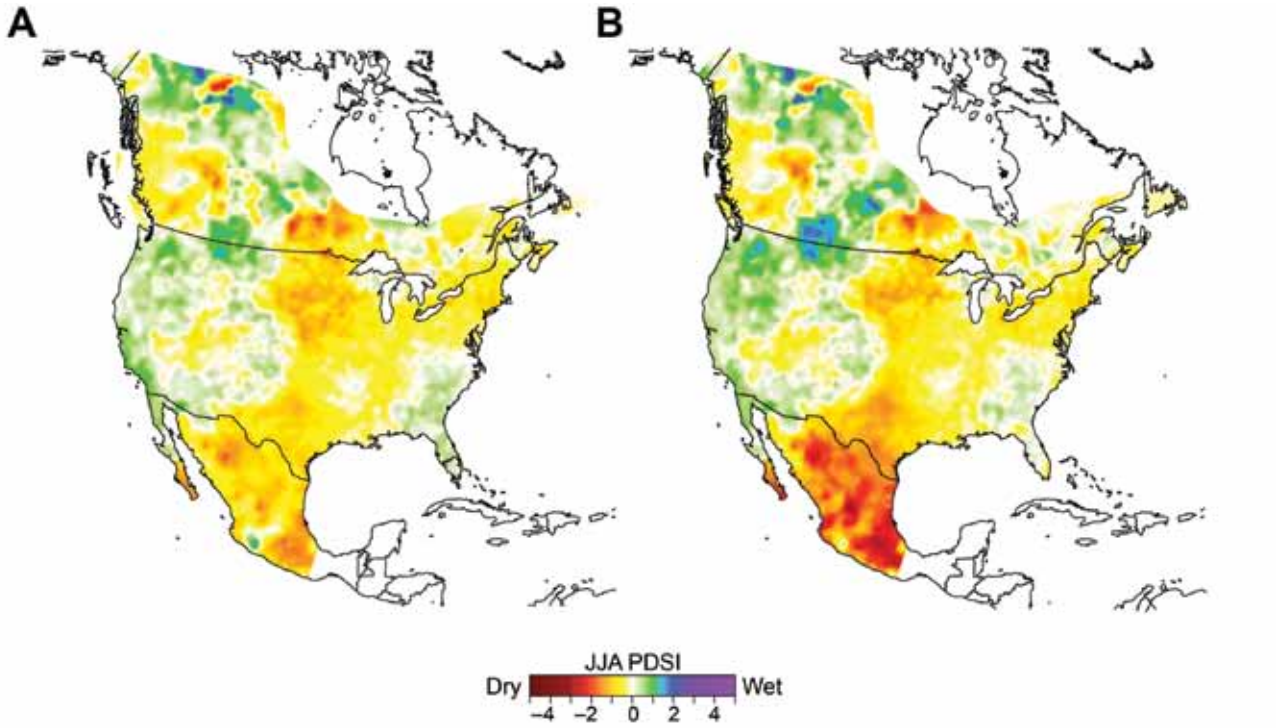


Figure 3. Tree-ring–reconstructed summer Palmer Drought Severity Index (PDSI) during A) 22 and B) 15 typhus epidemics in Mexico, 1665–1918. Drought conditions are indicated by negative values on the PDSI scale. Reconstructed summer PDSI values during the 15 typhus epidemic years with the most negative PDSI values for central Mexico are mapped in panel B. JJA, June–July–August.

The epidemic of 1861 coincided with drought and high grain prices in central Mexico. Historical sources agree that the epidemic was triggered by the mass movement of soldiers and starving refugees into Mexico City in search of food and relief. During the 1875–1877 epidemic, all of Mexico experienced severe food scarcity while a large wave of poor immigrants arrived in Mexico City. Mass migration of refugees into cities, spurred by food scarcity and the ongoing revolution, also may have played a role in the epidemic of 1915–1918 (J.N. Burns, unpub. data).

## Conclusions

Substantial evidence has been found for the role of drought in epidemic typhus during 1655–1918 in Mexico. The historical records implicate environmental refugees as the possible mechanism by which drought contributed to the spread of epidemic typhus during the 22 examined epidemics. However, this theory needs further investigation. Verification of the role of environmental refugees in the transmission of typhus during these 22 epidemics is complicated by a lack of clarity in the historical sources and by the interaction between politics, geography, and climate. The exact timing for the onset and withdrawal of drought, famine, or typhus epidemics as recorded in historical sources can be ambiguous. Gaps and contradictions in the historical records need to be resolved through a more detailed, multifocal historical analysis. In addition, most historical citations describing typhus outbreaks are from Mexico City, but tree-ring reconstructions indicate that drought was much more widespread in Mexico during these events. The true spatial extent of typhus during these 22 and perhaps other epidemics needs to be identified in the historical records of other localities.

The long-term collections of historical data have provided insight into epidemics of typhus during the era before antimicrobial drugs. Detailed chronology of typhus epidemics and tree-ring data are rich and unexhausted scientific resources but, unfortunately, both are threatened by inadvertent destruction by archival destruction and deforestation (*26*). Mexico has a long and detailed historical record and some of the utmost forest biodiversity in the world. The integration of historical epidemiologic data with proxy climate data from tree rings promises to improve understanding of the interactions between climate and society that result in epidemic disease in Mexico.

Ms Burns is a graduate student in the Department of Geosciences, University of Arkansas, Fayetteville, Arkansas. Her research interests are geospatial analysis and the interactions of climate, environment, and society.

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# Nontoxigenic *tox*-bearing *Corynebacterium ulcerans* Infection among Game Animals, Germany

**Tobias Eisenberg, Peter Kutzer, Martin Peters, Andreas Sing, Matthias Contzen, and Jörg Rau**

*Corynebacterium ulcerans* may cause diphtheria in humans and caseous lymphadenitis in animals. We isolated nontoxigenic *tox*-bearing *C. ulcerans* from 13 game animals in Germany. Our results indicate a role for game animals as reservoirs for zoonotic *C. ulcerans*.

The *Corynebacterium* species *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* form the *C. diphtheriae* group, as shown by 16S rRNA gene sequence analysis (*1*). Strains of this group carrying lysogenic β-corynephages might produce the *tox*-encoded diphtheria toxin (DT) (*2*). Moreover, *C. ulcerans* and *C. pseudotuberculosis* may produce phospholipase D, the major virulence factor involved in caseous lymphadenitis, which is a disease that mainly affects sheep, goats, and horses (*3*).

From a public health perspective, diphtheria is the most critical human disease attributed to coryneform bacteria (*3*). In recent years, cases of diphtheria caused by *C. ulcerans* have outnumbered those caused by *C. diphtheriae* (*4*). *C. diphtheriae* carriage is nearly exclusively restricted to humans; *C. ulcerans* is a zoonotic pathogen and has been found in various animal species that have contact with humans (*5*). *C. ulcerans* is most closely related to *C. pseudotuberculosis*, and distinction between these species is often difficult when using standard bacteriological methods (*5*). The aim of this study was to comprehensively characterize 13 *C. ulcerans* strains isolated from game animals in Germany.

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## The Study

Strains of *C. ulcerans* were isolated during routine bacteriological investigations in conjunction with necropsies of wild animals that were found dead or that had suspicious lesions during 1997–2013. Isolates of coryneform bacteria were subjected to conventional biochemical tests (*3*), and were evaluated after prolonged incubation at 37°C for as long as 14 days. For further characterization, commercial tests API Coryne and VITEK2-compact with cards for coryneform bacteria and corynebacteria and anaerobes (bioMérieux, Nürtingen, Germany) were used according to the manufacturer's instructions.

We conducted the reverse CAMP test by using *Staphylococcus aureus* American Type Culture Collection (ATCC [Manassas, VA, USA]) 25923 and the CAMP test by using *Rhodococcus equi* ATCC 33701 according to standard procedures on Columbia sheep blood agar (Oxoid, Wesel, Germany) (*3*). We determined DT production using a modified Elek test (*6*); we used *C. diphtheriae* NCTC 10648 and *C. diphtheriae* NCTC 10356 as positive and negative

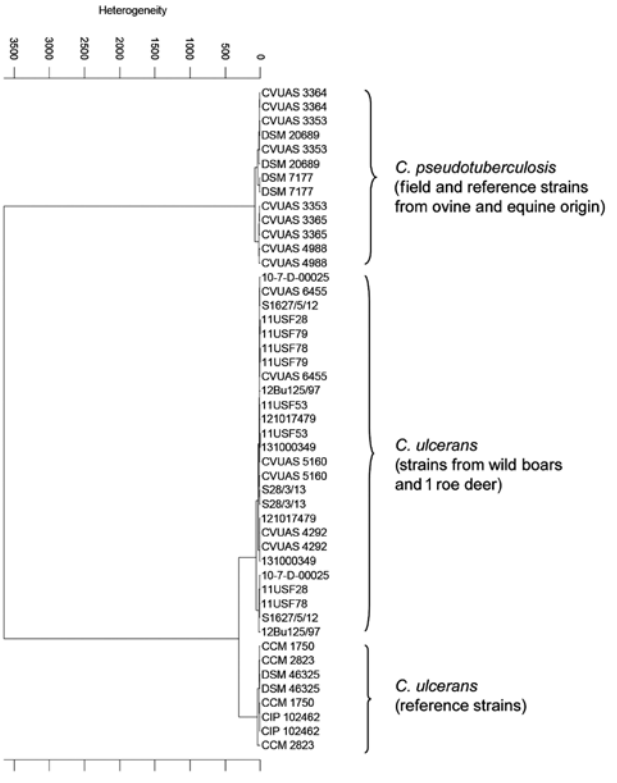


Figure 1. Cluster analysis of respective spectra obtained by Fourier-transform infrared-spectroscopy by using OPUS Software version 4.2 (BrukerOptics, Ettlingen, Germany). In each case, 2 infrared spectra of isolates from game animals and a selection of several *Corynebacterium ulcerans* and *C. pseudotuberculosis* strains were used for calculation by using the Ward algorithm. The dendrogram depicts the arrangement of isolates in groups according to their spectral differences.

Table 1. Origin of nontoxigenic *tox*-bearing *Corynebacterium ulcerans* field strains among game animals and gross pathology results from necropsies, Germany

| Case no. | Isolate ID | Year of isolation | State/district of origin | Host species | Circumstances of death; gross pathology results | Source |
|---|---|---|---|---|---|---|
| 1 | Bu125/97 | 1997 | North Rhine-Westphalia/ Siegen-Wittgenstein | Wild boar | Meat-inspection; lamellar lymph node abscess | This study |
| 2 | CVUAS 4292 | 2009 | Baden Wuerttemberg/Enz | Wild boar | Found dead; multiple lamellar lymph node abscesses; multiple hypertrophic lymphangitis | (*5*) |
| 3 | CVUAS 5160 | 2009 | Baden Wuerttemberg/ Main-Tauber | Wild boar | Shot; superficial cervical lymph nodes greatly enlarged; abscess of *Ln. mandibularis* | (*5*) |
| 4 | CVUAS 6455 | 2010 | Baden Wuerttemberg/ Aalen | Roe deer | Moribund; grapefruit-sized abscess of or near left *Ln. cervicalis superficialis* | (*8*) |
| 5 | 10–7-D-00025 | 2010 | Hesse/Lahn-Dill | Wild boar | Shot; female; lamellar thoracic plum-sized lymph node abscess | This study |
| 6 | 11USF28 | 2011 | Brandenburg/ Havelland | Wild boar | Found dead; male, ≈2 y old; subcutaneous abscess | This study |
| 7 | 11USF53 | 2011 | Brandenburg/ Havelland | Wild boar | Shot; female, ≈3 y old; lung abscess | This study |
| 8 | 11USF78 | 2011 | Brandenburg/ Havelland | Wild boar | Shot; female, ≈1 y old; subcutaneous abscess | This study |
| 9 | 11USF79 | 2011 | Brandenburg/ Havelland | Wild boar | Shot; male, ≈1 y old; subcutaneous abscess | This study |
| 10 | 121017479 | 2012 | Hesse/ Marburg | Wild boar | Shot; some milium- to pea-sized solid grayish abscesses with dystrophic central calcification in diaphragmatic peritoneum | This study |
| 11 | S1627/5/12 | 2012 | North Rhine-Westphalia/ Siegen-Wittgenstein | Wild boar | Shot; 1 y old; multiple lamellar abscesses in cervical and pulmonal lymph nodes | This study |
| 12 | S28/3/13 | 2013 | Hesse/Bad Hersfeld | Wild boar | Shot; 2 y old; isolate from teat abscess; multiple lamellar abscesses in cervical lymph nodes | This study |
| 13 | 131000349 | 2013 | Hesse/Odenwald | Wild boar | Found dead; female, ≈1 y old; some cherry-sized subcutaneous lymph node abscesses | This study |

controls, respectively; and performed a cytotoxicity assay using Vero cells (*7*). The *rpoB* and *tox* genes were partially amplified by using primer pairs C2700F/C3130R and DT1/DT2, respectively, as described (*5*).

PCR products were purified for sequence analysis by using the Double Pure Combi Kit (Bio&SELL, Nürnberg, Germany). Both strands of the *rpoB* and *tox* PCR products were sequenced by Microsynth (Balgach, Switzerland) by using the amplification primers. Sequence analysis was performed by using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) sequence analysis tool. Additionally, coryneform isolates in which *C. ulcerans* was suspected were analyzed by using Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) and by using Biotyper version 3.3.1.0 (BrukerBiotyper; BrukerDaltonics, Bremen, Germany). The database used (DB 4613) comprised spectra from 71 *Corynebacterium* species including *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. For Fourier-transform infrared (FT-IR) spectroscopy, bacterial isolates were harvested and prepared as described (*5*). IR spectra were recorded by using an FT-IR spectrometer (Tensor 27 with High Throughput Screening eXTension HTS-XT module) and OPUS software version 4.2 (Bruker Optics, Ettlingen, Germany). IR spectra of isolates from game animals and selected *C. ulcerans* and *C. pseudotuberculosis* strains were compared by cluster analysis by using the second derivation of vector normalized spectra (*8*). The dendrogram obtained depicts the arrangement of isolates in groups according to their spectral differences (Figure 1).

In total, 13 strains of *C. ulcerans* were isolated from 12 wild boars and 1 roe deer in 4 states of Germany (Table 1). The bacteria grew from ≥1 delimited pseudotuberculosis-like caseous abscess, arranged in concentric layers and ranging from 0.1 to 10 cm in diameter (Figure 2). All strains had positive reactions by using a traditional CAMP test inoculated with *R. equi* and a reverse CAMP test inoculated with *S. aureus*, indicating phospholipase D activity (*3*).

Conventional biochemical tests showed congruent results for catalase activity, urea hydrolysis, and glucose acidification (positive) and for esculin hydrolysis and nitrate reduction (negative). Additional reactions and API and VITEK test results are shown in Table 2. All isolates

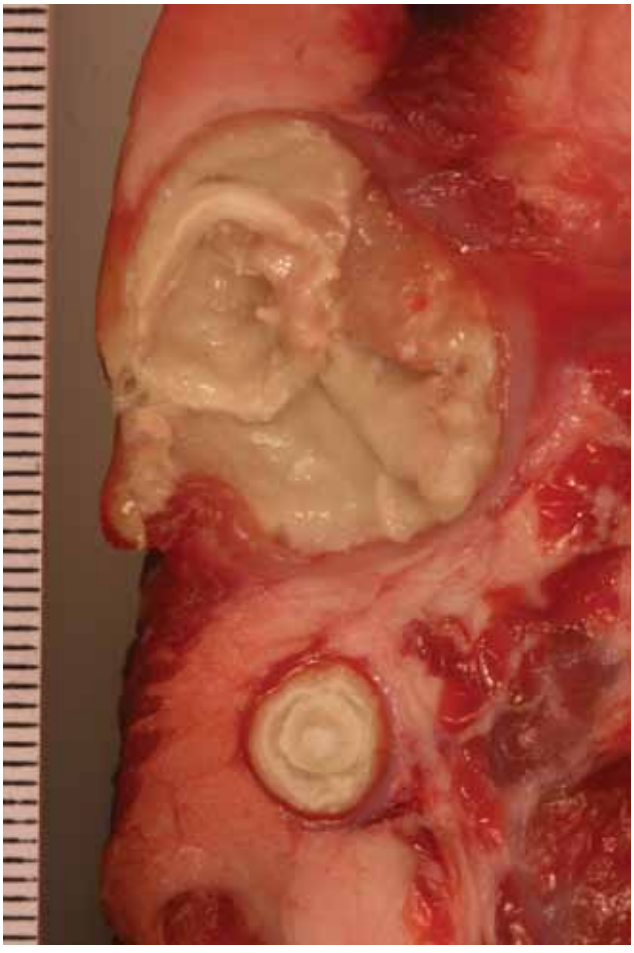

Figure 2. Pseudotuberculosis-like caseous abscesses caused by *Corynebacterium ulcerans* in wild boar S28/3/13. Scale is shown in millimeters.

were nontoxigenic *tox*-bearing (NTTB) strains as shown by positive *tox*-PCR, and negative Elek test and Vero cell cytotoxicity results. Partial *rpoB* and partial *tox* sequences for all 13 isolates were identical to those submitted to GenBank for *C. ulcerans* strain CVUAS 4292 (accession nos. GU818735 and GU818742, respectively [*5*]).

By using MALDI-TOF MS, all isolates were identified to the species level as *C. ulcerans* because they had score levels of 2.0–2.2. The comparison of the IR spectra of the 13 strains from game animals with a collection of reference strains showed a clear separation in 2 main branches for the 2 species *C. pseudotuberculosis* and *C. ulcerans* (Figure 1). Inside the *C. ulcerans* branch, all isolates from game animals clustered compactly together and were closely adjacent to a group of spectra formed by reference strains from humans.

## Conclusions

With respect to its zoonotic potential, *C. ulcerans* is one of the most notable members of the genus and was referred to as an emerging pathogen in 2011 (*9*). Numerous reports state there is zoonotic potential for contact with companion or farm animals, but proven transmission of *tox*-positive *C. ulcerans* strains is documented for only 4 cases, involving 2 dogs, 1 cat, and 1 pig (*8*).

Limited information is available concerning *C. ulcerans* infection in wild animals. To our knowledge, 3 reports regarding *tox*-positive *C. ulcerans* infection in wildlife have been published: 1 involved 2 European otters from 2 widely separated regions within the United Kingdom (*10*), and the other 2 reports described NTTB strains in 2 wild boars in 1 report and 1 roe deer in the other report; these 3 cases were in the same area of Germany (*5*,*8*). An additional report on *C. ulcerans* with unknown toxigenicity in wildlife pertains to an outbreak among 350 squirrels from Canada, 63 of which had clinical disease (*11*).

Here, we provide comprehensive data on 13 NTTB *C. ulcerans* strains from game animals in Germany. The finding of infected game in the center of Middle Europe suggests an even wider occurrence and distribution in other European countries. Misdiagnoses of *C. ulcerans* isolates as *C. pseudotuberculosis* in the past because of similar pathology and similar phenotype cannot be excluded. Our finding of *C. ulcerans* in a wild boar specimen from 1997 could indicate that this pathogen has not only recently infected wildlife.

As also shown in this study, biochemical differentiation between *C. ulcerans* and *C. pseudotuberculosis* might be problematic, and basic conventional tests may not properly discriminate between the 2 species (*3*). By using the standardized systems API Coryne and VITEK2-compact for coryneform bacteria, erroneous identification was made of most isolates (10 and 11 cases, respectively) from game animals as *C. pseudotuberculosis*. For correct understanding of epidemiology and host range and for unequivocal determination of the involved pathogen to species level, additional methods such as FT-IR and MALDI-TOF MS or DNA sequencing should be used. Because partial *rpoB* sequencing is more discriminatory than 16S rDNA sequencing, a cutoff value of ≤95% similarity proved suitable for species identification within *Corynebacterium* (*12*) and also clearly enabled species identification in this study. Furthermore, partial *tox*- and *rpoB*-gene sequencing demonstrated a very close relationship between the 13 strains because no variations in these sequences were found (*8*).

Concerning the zoonotic potential for *C. ulcerans* strains from wildlife, there is no information available. With respect to wild boars infected with *C. ulcerans*, however, it is noteworthy that 3 diphtheria cases occurred in humans who had occupational contact with pigs (*13*,*14*).

Table 2. Variable biochemical characteristics of API Coryne and VITEK2-compact profiles (bioMérieux, Nürtingen, Germany) of 13 *Corynebacterium ulcerans* field strains from game animals, Germany*

| Isolate ID | Ability of isolate to metabolize carbohydrate | | | | | API Coryne profile (interpretation/ % ID) | VITEK2 CBC profile (interpretation/%) | VITEK2 ANC profile (interpretation)† |
|---|---|---|---|---|---|---|---|---|
| | Maltose | Mannitol | Sucrose | Trehalose | Xylose | | | |
| Bu125/97 | – | – | – | + | – | 0 011 324 (Cps/99.5) | 01030140402010 (Cps/96) | 2123020000405 (Cul, Cje) |
| CVUAS 4292 | + | – | + | + | + | 0 041 725 (invalid) | 01030140406010 (Cps, Cdi/ -) | 2123020000405 (Cul, Cje) |
| CVUAS 5160 | – | + | – | + | + | 0 001 304 (Cps/97.5) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje/) |
| CVUAS 6455 | – | + | + | + | – | 5 153 325 (invalid) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |
| 10–7-D-00025 | – | – | – | + | – | 0 011 324 (Cps/99.5) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |
| 11USF28 | – | + | – | + | + | 0 011 324 (Cps/99.5) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |
| 11USF53 | – | – | – | + | – | 0 011 324 (Cps/99.5) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |
| 11USF78 | – | – | – | + | – | 0 011 324 (Cps/99.5) | 01030140402010 (Cps 96) | 2123020000405 (Cdi, Cje) |
| 11USF79 | + | – | – | + | – | 0 011 324 (Cps/99.5) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |
| 121017479 | – | – | – | – | – | 0 011 324 (Cps/99.5) | 01430140402010 (Cps/ 92) | 2123020000405 (Cul, Cje) |
| S1627/5/12 | – | – | – | + | – | 0 001 324 (Cps/99.9) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |
| S28/3/13 | – | – | – | + (weak) | + | 0 111 324 (Cps/92.7, Cul/7.2) | 01020140402010 (Cps, Cma/ -) | 2123020000405 (Cul, Cje) |
| 131000349 | – | – | – | + (weak) | + | 0 011 324 (Cps/99.5) | 01030140402010 (Cps/ 96) | 2123020000405 (Cul, Cje) |

*% ID, probability of identification according to the evaluation by the manufacturer; CBC, coryneform bacteria; ANC, *Corynebacterium* and anaerobes; Cps, *C. pseudotuberculosis*; Cul, *C. ulcerans*; Cdi, *C. diphtheriae*; Cje, *C. jeikeium*; Cma, *C. macginleyi*.
†Probability of identification was not provided by the manufacturer for cases in which test results identified 2 organisms.

Lack of DT expression in *tox*-positive strains has been described (*7*). Nevertheless, it can be expected that DT-producing *C. ulcerans* strains might occur in game animals, providing a reservoir for this microorganism. Because the *C. diphtheriae* and *C. ulcerans* DT sequences are quite similar, it might be reasonable to offer diphtheria toxoid vaccination to persons who have direct contact with game animals to prevent diphtheria-like illness caused by *tox*-positive *C. ulcerans* (*4*).


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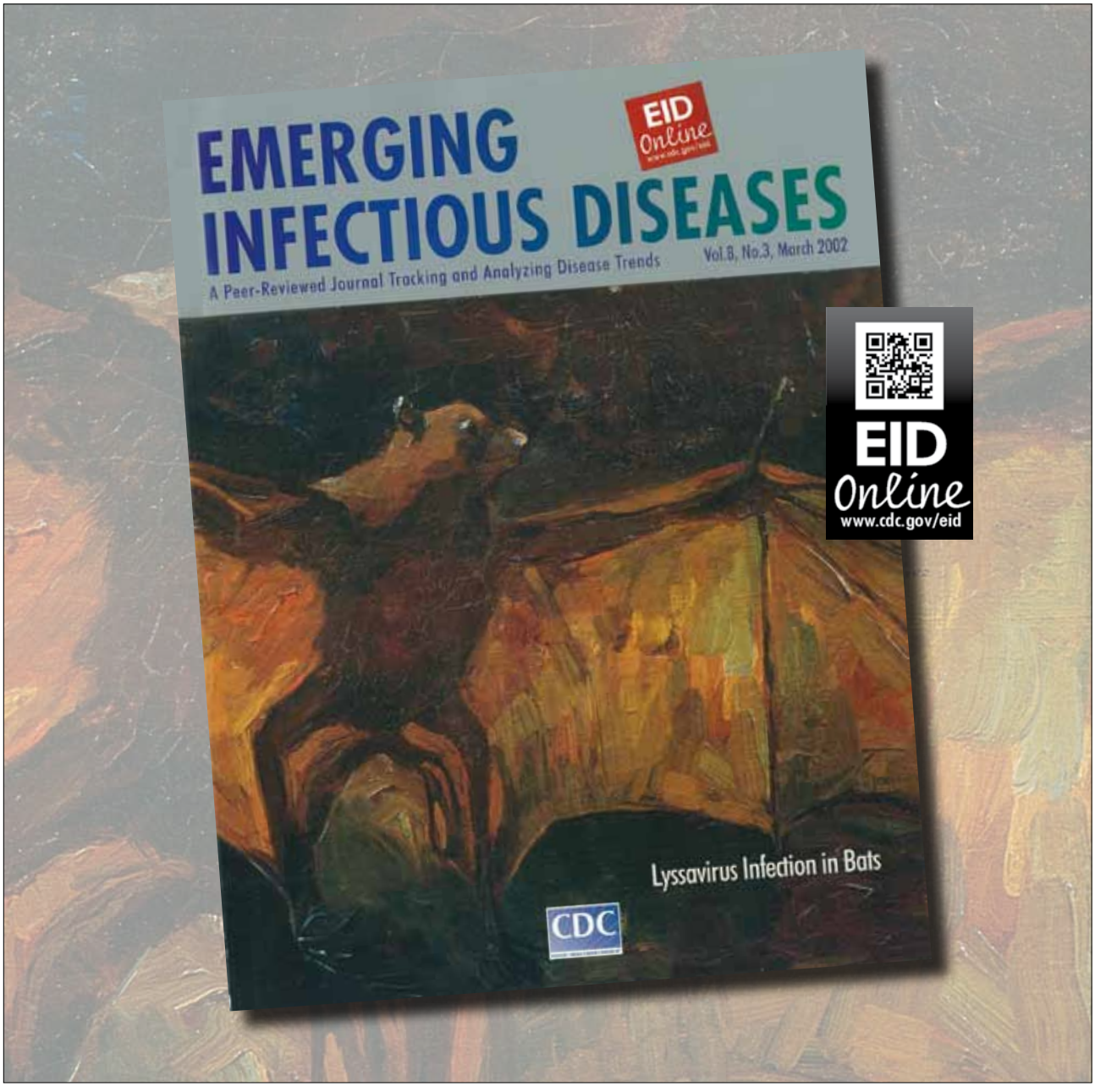

# Postmortem Diagnosis of Invasive Meningococcal Disease

**Alison D. Ridpath, Tanya A. Halse, Kimberlee A. Musser, Danielle Wroblewski, Christopher D. Paddock, Wun-Ju Shieh, Melissa Pasquale-Styles, Irini Scordi-Bello, Paula E. Del Rosso, and Don Weiss**

We diagnosed invasive meningococcal disease by using immunohistochemical staining of embalmed tissue and PCR of vitreous humor from 2 men in New York City. Because vitreous humor is less subject than other body fluids to putrefaction, it is a good material for postmortem analysis.

Invasive meningococcal disease is nationally reportable in the United States, and its case-fatality rate is 10%–14% (*1*). In New York City, New York, USA, every suspected or confirmed case is investigated to rapidly identify and recommend antimicrobial prophylaxis to close contacts. The risk for disease is highest during the initial 1–4 days after exposure. Fatal invasive meningococcal disease may go undiagnosed, which impairs prevention efforts and understanding of transmission. We describe 2 cases of serogroup C meningococcal disease diagnosed post mortem by PCR from vitreous humor and immunohistochemical (IHC) staining of tissues collected at autopsy.

## The Cases

### Case 1

In the fall of 2012, a man in his early 30s was found dead in his room by his family. He had attended a party and the next morning reported fever, chills, and general malaise (day 1). He was not seen again until day 3 when a family member found him unresponsive in his room. Emergency medical services personnel pronounced him dead. The case was reported to the New York City Office of the Chief Medical Examiner, and the medicolegal investigator noted that the man was in an early state of decomposition. No suspicious circumstances or evidence of external trauma or alcohol or drug use were present.

An autopsy performed on day 4 was remarkable for a purpuric rash more pronounced on the legs, arms, hands, and soles of feet (Figure 1). Early decomposition, commented on by the medicolegal investigator, was also noted at autopsy. Internal organs had a markedly soft consistency, but no other substantial abnormality was noted. The heart and valves were within normal limits without evidence of vegetation. The only substantial internal finding was a thin layer of purulent exudate on the leptomeninges. Cerebrospinal fluid (CSF) and blood samples could not be obtained. A limited amount of vitreous humor was collected; brain and other organ tissue samples collected were fixed in formalin.

The body was brought to the funeral home and embalmed on day 6. The medical examiner reviewing the case on day 6 became concerned that the patient might have died of meningococcal disease, and the examiner notified the New York City Department of Health and Mental Hygiene (DOHMH). DOHMH recommended that the medical examiner collect additional samples, specifically skin, for IHC. Three hours after embalming, skin samples were collected from areas of purpuric rash on the leg and stored in saline. Vitreous humor and skin samples were sent to Wadsworth Center laboratory of the New York State Department of Health (Albany, NY, USA) for testing by PCR, and tissue specimens (brain, lung, heart, liver, and kidney collected at autopsy and fixed in formalin and skin collected after embalming) were sent to the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) for IHC staining and PCR (*2*–*4*).

Skin and vitreous humor specimens were positive for *Neisseria meningitidis* serogroup C DNA by real-time PCR at Wadsworth Center (*2*). Gram-staining of brain and skin tissue (examination performed at CDC) showed gram-negative cocci and gram-positive bacilli in leptomeninges and vascular lumens. Widespread gram-positive bacilli were present in brain parenchyma without corresponding inflammation. Therefore, the gram-positive bacilli were deemed likely to represent postmortem bacteria overgrowth and did not contribute to an etiologic diagnosis. IHC assays that used a polyclonal anti–*N. meningitidis* group Y antiserum that is broadly reactive with serogroups A, B, C, W, and Y, and a specific monoclonal anti–*N. meningitidis* serogroup C antibody, revealed immunostaining in the leptomeninges, lung, and skin tissues (*3*,*4*). Results confirmed *N. meningitidis* serogroup C as the etiologic agent causing acute meningitis and systemic infection.

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Figure 1. Postmortem purpuric rash on sole of a man for whom invasive meningococcal disease was diagnosed after death (case 1), New York City, New York, USA.

Clinical, PCR, and IHC findings were all consistent with invasive meningococcal disease. All close contacts of the patient were located and given prophylaxis. No secondary cases were identified.

### Case 2

Approximately 2 months after the first case occurred, a man in his mid-30s was found dead in his apartment by his friends and police after he was reported missing from work. He had last spoken to friends 4 days previously when he reported a sore throat (day 1). The man had a history of HIV infection and crystal methamphetamine use, which was confirmed by urine toxicology at autopsy.

Autopsy was performed on day 6. The patient had marked putrefactive skin changes on the left side of the body, consistent with his postmortem position, and visible purpura on the right side of the torso and lower extremities but not on palms or soles. Flattening of cerebral gyri with apparent focal subarachnoid purulent exudate was also reported. Cerebral cortex and leptomeninge tissues were cultured and grew gram-positive rods and mixed flora. Tissue samples of lung, liver, spleen, kidney, and pancreas were fixed in formalin and sent to CDC. Vitreous humor and a swab of the leptomeninges collected at autopsy were sent to Wadsworth Center.

At Wadsworth Center, testing of the vitreous humor by real-time PCR showed *N. meningitidis* serogroup C DNA, *Haemophilus influenzae* DNA, *Streptococcus agalactiae* DNA, and *Staphylococcus aureus* DNA (*2*). As in case 1, the bacteria other than *N. meningitidis* were thought to represent postmortem bacteria overgrowth and did not contribute to etiologic diagnosis. At CDC, *N. meningitidis* DNA was extracted and amplified by PCR from formalin-fixed paraffin-embedded brain, liver, lung, spleen, and kidney tissue (*4*). Although multiple tissues showed marked autolysis, distinct and specific IHC staining for *N. meningitidis* serogroup C was identified within blood vessels and in neutrophilic infiltrates in the leptomeninges (Figure 2) (*3*,*4*). The results confirmed *N. meningitidis* serogroup C as the etiologic agent causing acute meningitis and systemic infection. DOHMH did not learn about the case until after it was too late to administer prophylaxis to close contacts, but no known secondary cases were identified.

## Conclusions

Suspecting and diagnosing meningococcal disease early is critical for initiating timely prophylaxis and preventing secondary cases. The 2 cases described here posed multiple challenges for diagnosing invasive meningococcal disease. In both cases, CSF and blood samples, where *N. meningitidis* is typically identified, were unavailable. Given the patients' unattended deaths and delayed discovery, putrefaction had set in and tissue samples were fairly decomposed. Additional bacteria were identified through real-time PCR or culture, further complicating the diagnosis for both cases.

*N. meningitidis* is a fastidious organism that frequently undergoes autolysis, although it has been found in CSF by PCR up to 10 days postmortem (*5*,*6*). Vitreous fluid has been described as a useful specimen for postmortem analysis because the eye is isolated and the fluid is less subjected to contamination or purification (*7*). *N. meningitidis* has been isolated from vitreous humor of living patients, usually in conjunction with symptoms of meningococcal endopthalmitis (*8*,*9*). We have demonstrated that postmortem diagnosis of *N. meningitidis* from vitreous humor and IHC staining ≤3 days after death is possible. This testing might

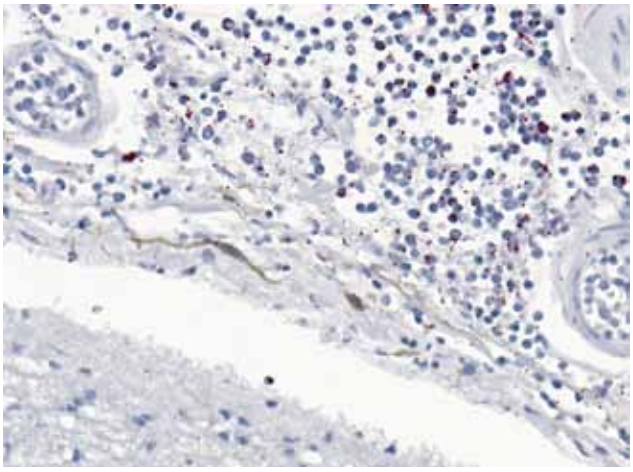

Figure 2. Immunostaining of *Neisseria menigitidis* in meninges of a man for whom invasive meningococcal disease was diagnosed after death (case 2), New York City, New York, USA. Naphthol fast red substrate with light hematoxylin counterstain. Original magnification ×25.

prove a useful option for medical examiners and public health officials for diagnosing suspected meningococcal disease when blood and CSF are unavailable for testing.


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Dr Ridpath is a CDC Epidemic Intelligence Service officer assigned to the New York City DOHMH. Her research interests include infectious disease epidemiology, specifically tropical medicine.


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# Urban Epidemic of Dengue Virus Serotype 3 Infection, Senegal, 2009

**Ousmane Faye, Yamar Ba, Oumar Faye, Cheikh Talla, Diawo Diallo, Rubing Chen, Mireille Mondo, Rouguiétou Ba, Edgard Macondo, Tidiane Siby, Scott C. Weaver, Mawlouth Diallo, and Amadou Alpha Sall**

An urban epidemic of dengue in Senegal during 2009 affected 196 persons and included 5 cases of dengue hemorrhagic fever and 1 fatal case of dengue shock syndrome. Dengue virus serotype 3 was identified from all patients, and *Aedes aegypti* mosquitoes were identified as the primary vector of the virus.

Dengue is an arboviral disease transmitted by *Aedes* spp. mosquitoes and caused by 4 serotypes of dengue virus (DENV): DENV-1, DENV-2, DENV-3, and DENV-4. DENV belongs to the family *Flaviviridae*, genus *Flavivirus* (*1*). More than 2.5 billion persons worldwide are considered at risk for dengue (*2*); the number of dengue infections per year has been estimated at 390 million, although only 96 million are symptomatic (*3*).

Most dengue infections occur in urban areas in tropical and subtropical regions, but imported cases have been reported in nontropical regions. During October 2009, imported DENV-3 infections were diagnosed in Turin, Italy (*4*), and Marseille, France (*5*), from patients returning from the Louga and Thies regions in Senegal (Figure 1). DENV-specific IgM and/or RNA were also detected in 5 persons living in Dakar, Senegal, who were suspected to have dengue. We report results from investigation of the 2009 dengue epidemic in Senegal.

## The Study

During October 2009–January 2010, a total of 696 serum samples were collected from persons in Senegal who were suspected to have dengue. A suspected dengue case was defined as fever and ≥2 of the following: myalgia, arthralgia, headache, and rash. Most samples were collected in Dakar (n = 606) and Thies (n = 87) (Table, Appendix, wwwnc.cdc.gov/EID/article/20/3/12-1885-T1.htm). In Dakar, samples were collected from 9 neighborhoods: Plateau (n = 202), Almadies (n = 157), Grand Dakar (n = 117), Parcelles Assainies (n = 82), Pikine (n = 13), Guediawaye (n = 15), Niayes (n = 5), Thiaroye (n = 10), and Rufisque (n = 5) (Figure 1). Samples were tested by real-time reverse transcription PCR and ELISA for virus genome and IgM, respectively.

In conjunction with human testing, mosquito sampling was performed during December 2009 in households with confirmed dengue cases, and entomologic risk indexes (i.e., Breteau and container indices [*6*]) were evaluated. DENV isolation and identification was performed on homogenized mosquitoes and human serum samples injected into mosquito cell lines, as described (*7*).

Partial DENV envelope protein coding regions were amplified by reverse transcription PCR (*8*), purified after electrophoresis from agarose gels, and sequenced for dengue serotype identification and phylogenetic analyses. A total of 196 (28.2%) suspected dengue cases were confirmed as dengue. Among confirmed case-patients, 45.9% (90/196), 33.0% (65/196), 10.2% (20/196), 10.2% (20/196), and 0.5% (1/196) belonged to the Senegalese, Lebanese, Lusophone, French, and Chinese communities, respectively; the Lusophone community includes persons from Cape Verde and Guinea-Bissau who live in Senegal. The ratio of confirmed to suspected cases was 17.8% (90/504), 66.3% (65/98), 51.2% (20/39), 38.4% (20/52), and 25.0% (1/4) for the Senegalese, Lebanese, Lusophone, French, and Chinese communities, respectively.

Among the 196 confirmed case-patients, 31 were hospitalized; 5 were found to have dengue hemorrhagic fever (DHF), and 1 died of dengue shock syndrome. The M:F sex ratio for the case-patients was 0.83 (89 male, 107 female), and the median age was 31 years (range 1–93).

The fatal case was in a 71-year-old Lebanese woman hospitalized in a private clinic in Dakar on October 30 with a 4-day history of fever; she had severe thrombocytopenia and elevated transaminase levels. Despite treatment (voluven and adrenaline), she died of cardiac arrest. Disseminated intravascular coagulation was probably responsible for the hemorrhagic syndrome.

Of the 5 other patients (4 Lebanese and 1 Senegalese) who had hemorrhagic manifestations, all had fever, epistaxis, and melena (5/5) associated with thrombocytopenia (platelet count 50–90 × $10^9$/L) and leukopenia (leukocyte count 2.5–3.5 × $10^9$ cells/L); 4 reported headache, and 3 reported myalgia/arthralgia. All 5 patients were hospitalized and received transfusions of fresh frozen plasma, platelet concentrates, and other supportive treatments.

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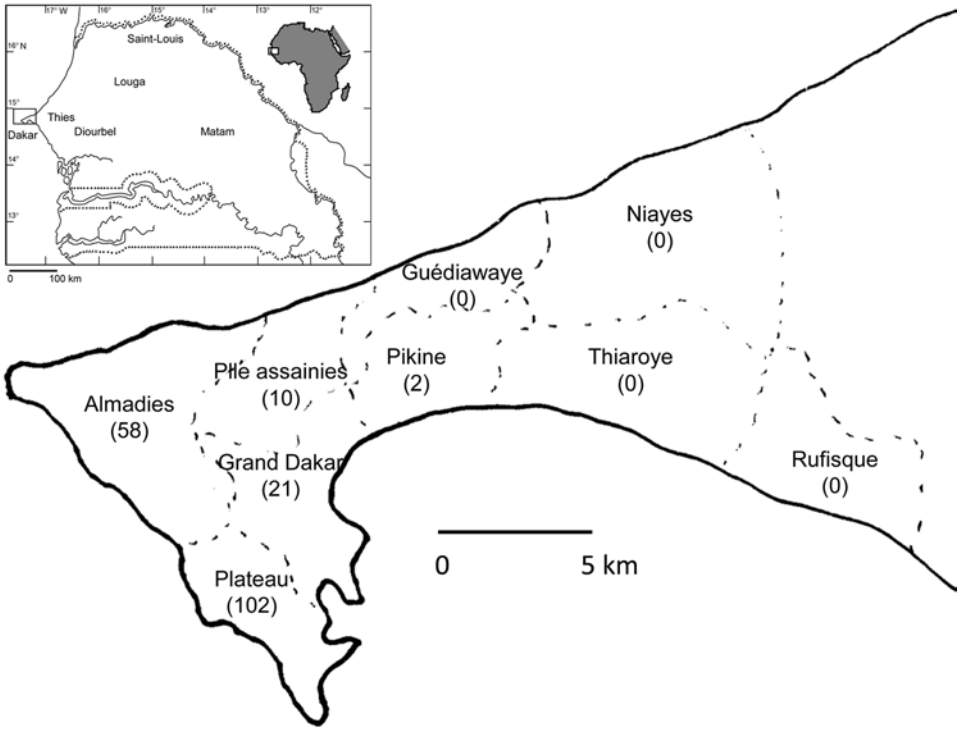


Figure 1. Geographic distribution of patients with confirmed dengue in the region of Dakar, Senegal. Number of patients is shown in parentheses. Inset shows location of Dakar in Senegal and in Africa.

Dengue-specific IgM and/or RNA were detected in serum samples collected 2 or 8 days after the onset of symptoms. All patients recovered and were discharged from the hospital after 8 or 10 days.

Using mosquito continuous cell lines, we recovered 49 DENV-3 isolates from confirmed case-patients. Phylogenetic analysis of sequences from mosquito and human samples revealed that DENV-3 genotype III closely related to isolates circulating in Côte d'Ivoire (2008) and China (2009) was circulating during the Senegal outbreak (Figure 2). Dates of illness onset of confirmed cases indicated that the outbreak started in late September 2009, peaked in mid-November, and declined in mid-December.

The entomologic investigation found high epidemic risk in all localities infested with DENV vectors. The Breteau index ranged from 6.6 to 195.2 in Dakar, 1.6 to 32.7 in Louga, and 1.1 to 14.9 in Thies, whereas the container indices ranged from 15 to 63.2 in Dakar, 5.3 to 15.2 in Thies, and 14.3 to 64.2 in Louga. A total of 5,730 mosquitoes were collected; these belonged to 8 species: *Aedes aegypti*, *Anopheles gambiae, Culex quinquefasciatus, Cx. tigripes, Cx. tritaeniorhynchus, Cx. antennatus, Cx. ethiopicus,* and *Cx. nebulosus*. Most mosquitoes were collected as adults, but 1,675 emerged from larvae that were collected in the field. *Cx quinquefasciatus* mosquitoes predominated, but *Ae. aegypti* mosquitoes, the only dengue vector collected, were found in all sites. DENV-3 was detected from 3 pools of mosquitoes collected in 2 neighborhoods of Dakar: Plateau (2 pools) and Parcelles Assainies (1 pool).

## Conclusions

An epidemic of DENV-3 occurred in Senegal during September–December 2009; of the 196 laboratory-confirmed cases, most (193) occurred in Dakar. This finding could indicate that transmission rates were higher in this urban area but may have been the result of bias in sample collection; dengue surveillance was less active at health facilities in other regions, which provided only 277 (39%) of the 696 samples collected from persons who were suspected to have dengue. Fever, headache, myalgia, vomiting, thrombocytopenia, and leukopenia were the most frequent signs and symptoms among patients with confirmed dengue, as described (*9*).

The proportion of DHF cases in this outbreak seemed to be high at 3% when compared with previous reports in the Americas from the 1980s through 2007, in which DHF rates ranged from 1.3% to 2.4% (*10*). However, our sample size was limited and lacks confirmatory power.

The percentage of confirmed dengue cases among suspected cases in different communities showed that persons in Senegalese communities were significantly less affected than those in Leebanese ($\chi^2 = 98.3$, df = 1; $p<0.0001$), Lusophone ($\chi^2 = 23.3$, df = 1; $p<0.0001$), and French ($\chi^2 = 11.3$, df = 1; $p<0.001$) communities. Moreover, 5 (83.3%) of the 6 cases with hemorrhagic manifestations occurred in the Lebanese community, which suggests that disease severity might be associated with community exposure.

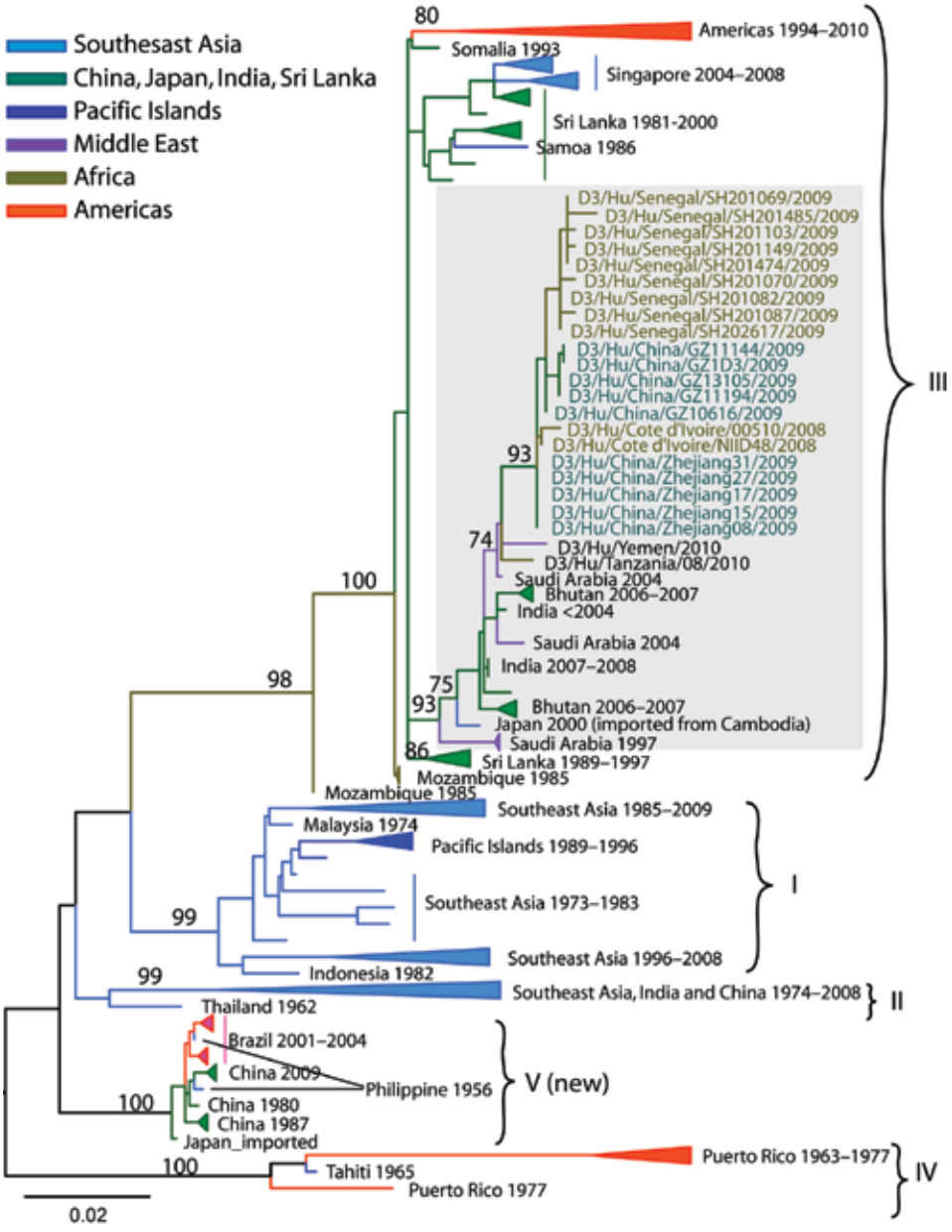


Figure 2. Maximum-likelihood phylogenetic tree of dengue virus serotype 3 (D3) sequences from Senegal compared with other sequences. The tree was constructed on the basis of an 885-bp segment of the envelope protein gene. Bootstrap values >70 are labeled next to the node. Sequences from different geographic areas are shown by different colors. Gray shading indicates sequences from Senegal and closely related strains. Scale bar indicates nucleotide substitutions per site.

*Ae. aegypti* mosquitoes were the most likely vector of DENV-3 transmission during this epidemic, given their association with DENV and the overlap of their distribution and abundance with the locations of dengue confirmed cases. *Ae. aegypti* mosquitoes are known to be a competent vector for DENV in West Africa (*11*) and could maintain DENV-3 through vertical transmission in Senegal, as described (*12*). The absence of suspected or confirmed dengue cases in Louga, despite the high density of *Ae. aegypti* mosquitoes, suggests that the first reported case-patient contracted the DENV infection elsewhere, possibly in Dakar, which he had visited several times.

The phylogenetic analysis of DENV-3 strains isolated during the outbreak suggests that they belong to genotype III and are closely related to DENV-3 isolated from Côte d'Ivoire and China in 2008 and 2009, respectively (*13*). Hence, the strain responsible for this outbreak may have been introduced into Senegal by travelers from Asia or from Côte d'Ivoire.

Our findings suggest that increased urban dengue activity is plausible in Senegal. Given ongoing population growth, explosive urbanization, infrastructure building, and international travel, dengue surveillance and preparedness should be reinforced. Furthermore, phylogenetic

studies incorporating more DENV-3 strains would shed light on the origins of this DENV-3 outbreak.


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# Role of *Waddlia chondrophila* Placental Infection in Miscarriage

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*Waddlia chondrophila* is an intracellular bacterium suspected to cause human and bovine abortion. We confirmed an association between antibodies against *W. chondrophila* and human miscarriage and identified this organism in placenta or genital tract of women who had had miscarriages. These results suggest a possible role of *W. chondrophila* infection in miscarriage.

Approximately 25% of pregnant women will experience at least 1 miscarriage (*1*,*2*). However, a cause is identified for only 50% of cases (*3*,*4*). Intracellular bacteria, which do not grow on media routinely used to isolate human pathogens from clinical samples, represent possible agents of miscarriage of unexplained etiology (*4*,*5*).

*Waddlia chondrophila*, a *Chlamydia*-related bacterium first identified in samples of bovine abortion tissues, has been associated with human miscarriages (*6*,*7*). In a study of 438 serum samples from women attending a recurrent-miscarriage clinic, seroprevalence of *W. chondrophila* was higher for women who had sporadic (31.9%) and recurrent (33.0%) miscarriages than for women who had uneventful pregnancies (7.1%; p<0.001) (*6*).

To further investigate the role of *W. chondrophila* in human miscarriage, we studied 386 women who had had miscarriages or uneventful pregnancies. In addition to serologic analysis, we used PCR and immunohistochemical analysis to detect *W. chondrophila* in placenta and vaginal samples.

## The Study

During 2006–2009, a total of 386 women were prospectively enrolled from the obstetrical ward of the University Hospital of Lausanne (Table 1) (*8*). The miscarriage group was composed of 125 women given a diagnosis of an acute episode of miscarriage in the emergency gynecology unit. The control group was composed of 261 women attending a labor ward, having uneventful pregnancies, and having no history of miscarriage, stillbirth, or preterm labor. Age, black race, and number of lifetime sex partners were different between both groups.

Immunofluorescence testing was performed by using *W. chondrophila* as antigen as described (*6*). Eighty-four women had antibodies against *W. chondrophila* as demonstrated by positive immunofluorescence against total immunoglobulin (Table 1). Among them, 67 women had IgG titers ≥1:64 and 6 women had IgM titers ≥1:32 against *W. chondrophila* (FluolineG or FluolineM; bioMérieux, Marcy l'Etoile, France). IgG seroprevalence was higher among women who experienced miscarriage (23.2%) than among women who experienced an uneventful pregnancy (14.6%; p = 0.044) (Table 2). When women with and without antibodies against *W. chondrophila* were compared, their age, contact with animals, education, number of previous sex partners, previous contraceptive use, and place of residence (countryside/city) were not associated with a positive serologic result for *W. chondrophila*. However, a multivariate logistic regression model indicated that black women were more likely to have antibodies against *W. chondrophila* (odds ratio [OR] 3.15, 95% CI 1.39–7.16).

As reported (*8*), we observed an association between miscarriage and *Chlamydia trachomatis* IgG seropositivity. The association between *W. chondrophila* miscarriage and seropositivity remained significant even when adjusted for *C. trachomatis* serostatus and vice versa. In a multivariate logistic regression adjusted for both variables, *C. trachomatis* and *W. chondrophila* seropositivity remained independently associated with miscarriage (OR 2.42, 95% CI 1.22–4.79 and OR 1.87, 95% CI 1.08–3.22, respectively).

After extraction of DNA by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), we tested all vaginal swab specimens and placenta samples by using a 16S rRNA *Waddlia* spp.–specific real-time PCR as described (*9*). No PCR inhibition was observed. Thirty-two samples (20 vaginal swab specimens and 12 placenta samples) were positive; no sample being positive in both types of samples. Ten of these positive PCR samples were from women who had had miscarriages; 9 of the 10 vaginal swab specimens had a positive PCR result (Tables 1, 2). Two of these 10 patients who had had miscarriages had IgG against *W. chondrophila* (patients 36 and 140). Patient 36 had the highest IgG titer (1,024) of the 386 women. Among the control group, 3 patients had IgG against *W. chondrophila* (titer ≥64). Among these women, 1 had IgG and IgM against *W. chondrophila* and 1 had only IgM against *W. chondrophila* (titer 32).

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Table 1. Characteristics of women, by miscarriage history, tested for infection with *Waddlia chondrophila**

| Characteristic | Control, n = 261 | Miscarriage, n = 125 | p value† |
|---|---|---|---|
| Age, y, ± SD | 31.5 ± 5.0 | 33.3 ± 6.1 | 0.002 |
| Race | | | <0.001 |
| White | 217 (84.8) | 69 (71.9) | |
| Black | 20 (7.8) | 21 (21.9) | |
| Asian | 19 (7.4) | 5 (5.2) | |
| Other | 0 | 1 (1.0) | |
| No. lifelong sex partners | | | 0.031 |
| 1 | 58 (22.2) | 37 (29.6) | |
| 2–3 | 43 (16.5) | 24 (19.2) | |
| 4–6 | 45 (17.2) | 10 (8.0) | |
| >6 | 36 (13.8) | 10 (8.0) | |
| Unknown | 79 (30.3) | 44 (35.2) | |
| *Waddlia spp.*–positive serologic result | | | |
| Total Ig ≥64 | 47 (18.0) | 37 (29.6) | 0.010 |
| IgG ≥64 | 38 (14.6) | 29 (23.2) | 0.044 |
| IgM 32 | 5 (1.9) | 1 (0.8) | 0.669 |
| *Waddlia* spp.–positive PCR result | | | |
| Vaginal swab specimen | 11 (4.2) | 9 (7.2) | 0.226 |
| Placenta | 11 (4.2) | 1 (0.8) | 0.113 |
| *Waddlia* spp.–positive immunohistochemical result | 1 (0.4) | 2 (0.8) | |
| *Chlamydia trachomatis* serologic result | | | |
| IgG positive | 19 (7.3) | 19 (15.2) | 0.018 |
| IgA positive | 10 (3.8) | 10 (8.0) | 0.091 |
| IgG and IgA positive | 7 (2.7) | 9 (7.2) | 0.037 |

*Adapted from Baud et al. (*7*,*8*). Values are no. (%) unless otherwise indicated.
†Statistical analysis was performed only for categorical variables.

All placenta specimens were examined by a pedopathologist (Table 2; Figure 1, panels A–D). Samples from the 10 women who had miscarriages and positive PCR results showed various histologic features, including deciduitis, chorioamnionitis, and plasmocytes in the decidua, which are compatible with chronic endometritis. Two of the samples showed standard histologic results.

Placentas from the 32 PCR-positive women and 10 PCR-negative controls were tested for *W. chondrophila* by using immunohistochemical analysis with a specific rabbit polyclonal antibody as described (*10*). Three placentas showed positive cells (Table 2; Figure 1, panels E–H). Patients 523 and 535, who had had miscarriages, had positive serologic results for total Ig but negative results for IgG and IgM (Table 2). Patient 250 was a woman who had had an uneventful pregnancy and who had positive PCR results for a vaginal swab specimen but negative serologic results. Immunohistochemical analysis showed that *W. chondrophila* infects mainly cells of the glandular epithelium; *W. chondrophila* was not found in endothelial cells (Figure 1).

Five women showed strong evidence of *W. chondrophila* infection, which was confirmed by ≥2 diagnostic tests (Figure 2). Thus, 2 women who had had a miscarriage had IgM and IgG (titer 32) and positive PCR results. Three other women (2 who had had miscarriages and 1 control) showed positive results by PCR and immunohistochemical analysis. Moreover, 31 other women showed some evidence of acute infection (i.e., 27 with a positive PCR result and/or 4 with IgM against *W. chondrophila*).

## Conclusions

Higher seroprevalence in the miscarriage group confirmed the association between miscarriage and *W. chondrophila* seropositivity observed in a study that investigated a population in London, UK (*6*). We also identified *W. chondrophila* DNA in the placenta and vagina of 32 women, including 10 who had had miscarriages. Among these 10 whose PCR result was positive, 4 were considered as having confirmed cases of infection because they also had positive serologic (n = 2) or immunohistochemical (n = 3) results. *W. chondrophila* in human tissue indicates that this intracellular bacterium might grow or persist within placental cells and might damage the placenta (*11*). The underlying mechanism of *Waddlia*-associated miscarriage may involve bacterial proteins, such as heat-shock protein 60, or production of inflammatory cytokines, such as tumor necrosis factor-α (*5*).

Detection of *W. chondrophila* in the vagina indicates that the infection might have originated after vaginal colonization. However, no association between sexual activity, use of condoms, and positive serologic results for *W. chondrophila* was reported in a study (seroprevalence 8.3%) of 517 young men in Switzerland (*12*).

We identified *W. chondrophila* in the human genital region. However, entry could occur at another site. *W. chondrophila* DNA has also been detected in sputa of patients with pneumonia (*9*,*13*), or other respiratory tract infections could disseminate to the uterus through the bloodstream. In contrast to our previous study findings (*4*), seropositivity for *W. chondrophila* was not associated with contact with animals.

Table 2. Characteristics of 10 women who had had miscarriages and had positive results for *Waddlia chondrophila* by real-time PCR*

| Patient no. | Age, y/race | Gravida/ parity | No. pregnancy weeks | Total Ig titer | IgG titer | PCR result for vaginal swab specimen | PCR result for placental specimen | Histologic result | IHC result | Other etiology |
|---|---|---|---|---|---|---|---|---|---|---|
| 7 | 37/ white | 4/3 | 11.2 | 0 | 0 | – | + | No inflammation | – | None found |
| 36 | 3/7 white | 1/0 | 6 | 64 | 1,024 | – | – | PMN in decidua | – | None found |
| 140 | 34/ black | 1/0 | 6 | 64 | 128 | – | – | PMN and plasmocytes in decidua compatible with chronic endometritis | – | None found |
| 183 | 42/ white | 3/1 | 9 | 0 | 0 | + | – | PMN in decidua and glandular epithelium compatible with early infection | – | None found |
| 305 | 29/ white | 5/0 | 21 | 0 | 0 | + | – | CAM (PMN in chorion and extension of these inflammatory cells to amnios) | – | *Ureaplasma urealyticum* in vaginal swab specimen |
| 357 | 19/ Asian | 2/1 | 8 | 0 | 0 | + | – | Rare lymphocyte in decidua | – | *Brucella abortus* antibodies |
| 409 | 42/ other | 3/1 | 10 | 0 | 0 | + | – | PMN in subchorial fibrin and glandular epithelium compatible with early infection | – | HT |
| 459 | 29/ white | 3/1 | 9 | 0 | 0 | + | – | PMN and hemorrhagic necrosis | – | None found |
| 523 | 34/ other | 3/1 | 10.5 | 164 | 0 | + | – | No inflammation | + | *Chlamydia trachomatis* antibodies (PCR negative) |
| 535 | 35/ white | 3/1 | 10 | 164 | 0 | + | – | PMN in fibrin of decidua compatible with early infection | + | None found |

*IHC, immunhistochemical; +, positive; –, negative; PMN, polymorphonuclear cells; CAM, chorioamnionitis; HT, hyperthyroidism.

This prospective study confirmed an association between *W. chondrophila* seropositivity and miscarriage. Four (3.2%) of 125 women who had had miscarriages were positive by serologic analysis and PCR or by PCR and immunohistochemical analysis and were considered as having confirmed cases of infection. One (0.4%) *W. chondrophila* infection was documented by 2 diagnostic tests in a women in the control group who had not had a miscarriage ($p = 0.04$). These results suggest a strong association between *W. chondrophila* infection and miscarriage among women (*6*,*7*). When a *W. chondrophila*–associated miscarriage is suspected, we recommend performing PCR on placenta and vaginal swab specimens and serologic analysis.

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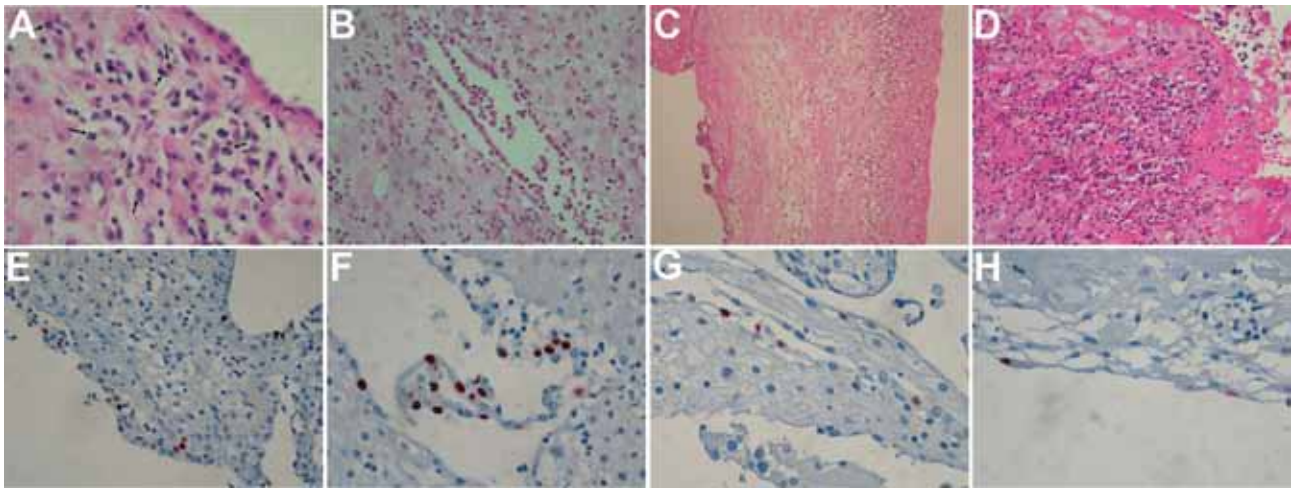


Figure 1. Histopathologic analysis of placentas from women tested for infection with *Waddlia chondrophila*. A) Patient 140, chronic endometritis with various inflammatory cells in the deciduas, including plasmocytes (arrows) (original magnification x600). B, Patient 183, polymorphonuclear cells (PMN) in a an endometrial gland (original magnification x400). C) Patient 305, chorioamnionitis with PMN extending from the chorion to the amnios (original magnification x200). D) Patient 535, PMN in the subchorial fibrin near the gestational sac (original magnification x400). Hematoxylin and eosin stain. Immunohistochemical analysis showing *W. chondrophila* in placental tissue. A rabbit polyclonal antibody directed against *W. chondrophila* was used at a dilution of 1:12,000. Detection was performed by using the ChemMate Kit (Dako, Glostrup, Denmark). Negative controls contained antibody diluent instead of primary antibody. Negative and positive control pellets were included as described (*10*). All highly positive cells were found in epithelium of endometrial glands. E) Patient 535 (miscarriage) (original magnification x400). F) Patient 535 (original magnification x600) G) Patient 523 (miscarriage) (original magnification x600). H) Patient 250 (control) (original magnification x600). 3-amino-9-ethylcarbazole/peroxidase stain and hematoxylin counterstain.

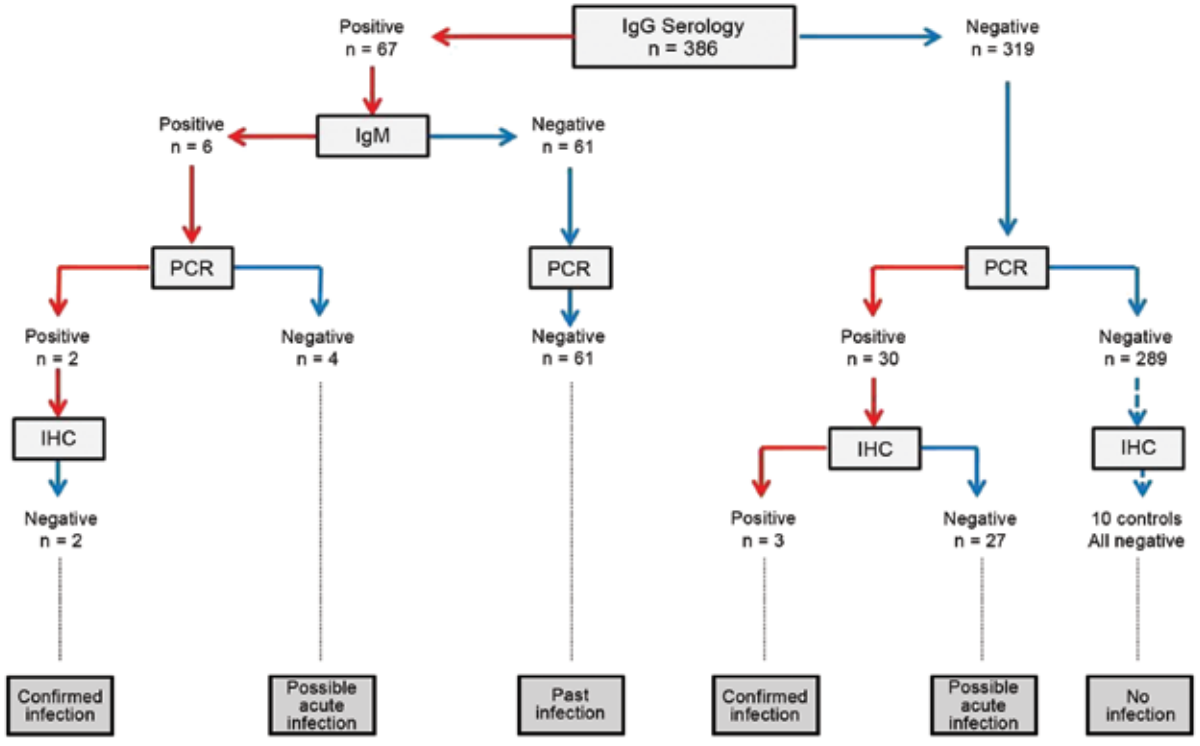


Figure 2. Decision tree for samples from placentas, which were used to screen for infection with *Waddlia chondrophila*. Of 386 women, a total of 5 had a confirmed infection, which was defined as a minimum of 2 independent positive *W. chondrophila*–specific test results, and 31 had evidence of acute current *W. chondrophila* infection. IHC, immunohistochemical analysis.

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# IgG Against Dengue Virus in Healthy Blood Donors, Zanzibar, Tanzania

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We conducted a seroprevalence survey among 500 healthy adult donors at Zanzibar National Blood Transfusion Services. Dengue virus IgG seroprevalence was 50.6% and independently associated with age and urban residence. These data will aid in building a surveillance, preparedness, and response plan for dengue virus infections in the Zanzibar Archipelago.

In eastern Africa, the available evidence indicates that dengue virus serotypes 1, 2, and 3 (DENV-1, -2, -3) are common causes of acute fever (*1*). A recent map of DENV transmission has shown that the virus could be transmitted in most eastern African countries, including mainland Tanzania and the Zanzibar Archipelago (*2*).

In 2010, a PROMED report raised concerns about DENV infections in Tanzania (*3*). That same year, travelers from Europe and Japan were found to be infected with DENV-3 after they returned from mainland Tanzania or Zanzibar (*4–6*). In Tanzania, seroprevalence rates for febrile outpatients in Tosomaganga (Iringa Region) and Pemba Island (Zanzibar) in 2007 (*7*) and in Moshi (Arusha Region) in 2007–2008 (*8*) were 1.8%, 7.7%, and 10.7%, respectively. To determine DENV circulation in the Zanzibar Archipelago, we assessed the seroprevalence of DENV among adult blood donors at the Zanzibar National Blood Transfusion Services (ZNBTS).

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## The Study

We conducted a cross-sectional seroprevalence survey at ZNBTS from September 20 to December 10, 2011. ZNBTS is located in Stone Town, the principal city of the Zanzibar Archipelago. Ethics approval was obtained from the Zanzibar Medical Research Ethical Committee. The sample size was calculated by using methods for proportion. The estimated prevalence was set at 50% because data were not available regarding the true prevalence of the infection in the area. Considering a population of ≈1,000,000 inhabitants and a confidence level of 95%, the sample size was set at 384 donors. Sample size was then increased to 500 donors to account for those lost to follow-up.

During the study period, all consecutive adult donors attending ZNBTS, who had been screened and selected for blood donation, were enrolled in the study. Donors were screened by serologic tests for hepatitis B virus, hepatitis C virus, HIV, and *Trepomena pallidum*; they were selected for blood donation if results of all screening tests were negative. A structured interview was conducted by using a close-ended questionnaire after the donor signed the informed consent form and before the screening.

From each enrolled person, 10 mL of venous blood was collected. After the screening tests, the remaining serum was divided into 2 aliquots: 1 was stored at -20°C at the sample processing site for performance of the IgG ELISA at Mnazi Mmoja Hospital in Unguja, Zanzibar, and 1 was dispatched to the L. Spallanzani National Institute for Infectious Diseases in Rome, Italy, for testing by immunofluorescence assay (IFA) for IgG. At the end of the collection phase, samples were tested by Panbio Dengue IgG Indirect ELISA kit (Inverness Medical Innovations Australia Pty Ltd, Sinnamon Park, Queensland, Australia) according to the manufacturer's instructions. A positive ELISA result was defined as having an index value >1.1. To compensate for the low specificity of the ELISA, we tested samples by IFA with homemade slides and a mix of uninfected and DENV-2 (New Guinea C strain)–infected Vero E6 cells. The diagnostic accuracy of the IFA has been described (*9*).

Donors were considered positive for IgG against DENV if results of both tests were positive. Discordant results were considered negative. All districts except the urban district were considered rural areas. Univariate association between DENV IgG positivity and donor characteristics was assessed by means of odds ratios (ORs) and 95% CIs, by $\chi^2$ for categorical values, and Student *t*-test for continuous variables. A multiple logistic regression model using a backward procedure was used. All variables were entered in the backward selection model, and a cutoff level of $p = 0.10$ was used for subsequent

Table 1. Univariate analysis of characteristics of 500 blood donors according to IgG positivity against dengue virus, Zanzibar, 2011*

| Characteristic | IgG positive, n = 253, no. (%) | IgG negative, n = 247, no (%) | Total, N = 500 | OR (95% CI) | p value |
|---|---|---|---|---|---|
| Age, mean (±SD) | 34 (±9) | 30 (±8) | 32 (±9) | | <0.001 |
| Age, for 5-y increase† | | | | 1.32 (1.19–1.47) | <0.001 |
| Age, y | | | | | |
| ≥36 | 57 (39.6) | 87 (60.4) | 144 | 1 | |
| ≤25 | 81 (43.8) | 104 (56.2) | 185 | 1.19 (0.76–1.85) | 0.444 |
| 26–35 | 115 (67.3) | 56 (32.7) | 171 | 3.13 (1.97–4.98) | <0.001 |
| Sex | | | | | |
| M | 243 (50) | 243 (50) | 486 | 1 | |
| F | 10 (71.4) | 4 (28.6) | 14 | 2.5 (0.77–8.07) | 0.126 |
| Work | | | | | |
| Yes | 172 (52.4) | 156 (47.6) | 328 | 1 | |
| No | 80 (47.1) | 90 (52.9) | 170 | 0.8 (0.55-1.16) | 0.254 |
| Persons in household | | | | | |
| 1-4 | 80 (47.1) | 90 (52.9) | 170 | 1 | |
| 5–8 | 129 (53.8) | 111 (46.2) | 240 | 1.3 (0.88–1.93) | 0.182 |
| ≥9 | 44 (48.9) | 46 (51.1) | 90 | 1.07 (0.64–1.79) | 0.779 |
| Bed net use | | | | | |
| Yes | 146 (49.2) | 151 (50.8) | 297 | 1 | |
| No | 107 (52.7 | 96 (47.3) | 203 | 1.15 (0.80–1.64) | 0.436 |
| Insecticide-spraying home | | | | | |
| Yes | 107 (53.5) | 93 (46.5) | 200 | 1 | |
| No | 146 (48.7) | 154 (51.3) | 300 | 0.82 (0.57–1.17) | 0.290 |
| Flu | | | | | |
| Yes | 40 (48.8) | 42 (51.2) | 82 | 1 | |
| No | 213 (51) | 205 (49) | 418 | 1.09 (0.67–1.75) | 0.719 |
| Resting water‡ | | | | | |
| Yes | 223 (50.7) | 217 (49.3) | 440 | 1 | |
| No | 30 (50.8) | 29 (49.2) | 59 | 1.00 (0.58–1.73) | 0.981 |
| Water storage§ | | | | | |
| Yes | 172 (48.3) | 184 (51.7) | 356 | 1 | |
| No | 82 (56.9) | 62 (43.1) | 144 | 1.39 (0.94–2.06) | 0.093 |
| Area of living | | | | | |
| Rural | 119 (39.4) | 183 (60.6) | 302 | 1 | |
| Urban | 134 (67.7) | 64 (32.3) | 198 | 3.18 (2.21-4.69) | <0.001 |

*OR, odds ratio.
†Age, 5-y increase is defined as the odds ratio for every 5 years of increase.
‡Resting water is defined as any presence of water resting around the house (i.e.. ponds, puddles).
§Water storage is defined as any container used to collect rain water with no mention of the size and type of container.

selections. Data management and analysis were performed by using STATA version 11 (StataCorp, College Station, TX, USA).

Five hundred persons consecutively attending ZNBTS were selected for blood donation and, therefore, were eligible to be enrolled in the study. Demographic characteristics of the participants are shown in Table 1. The mean age was 32 years; 97.2% of the participants were male. Most donors had a water storage container (71.2%) and/or resting water near their home (88.0%). Bed nets were used by 59.4% of the donors, but only 40% reported insecticide spraying at home.

Of the 500 blood samples, 253 (50.6%) were positive by both tests, 77 (15.4%) were positive by ELISA and negative by IFA, and 170 (34.0%) were negative by both tests. DENV IgG prevalence was 50.6% (95% CI, 46.2–54.9). Considering IFA as the reference standard, ELISA sensitivity and specificity were 100% and 68.8%, respectively. According to univariate analysis, DENV IgG–positive donors were significantly more likely to be older (OR 1.32, 95% CI 1.19–1.47) and live in urban districts (OR 3.18 95% CI 2.21–4.69) compared with DENV IgG-negative donors (Table 1). According to multivariate analysis, older age (adjusted OR [AOR] 1.42, 95% CI 1.27–1.61) and living in an urban district (AOR 4.09, 95% CI 2.72–6.17) were independently associated with DENV IgG positivity (Table 2). Moreover, borderline evidence indicated an association of positivity with the presence of resting water near the home (AOR 1.66, 95% CI 0.99–2.76).

## Conclusions

We found high DENV IgG seroprevalence (50.6%) in adult blood donors residing in the urban district. The presence of DENV IgG is independently associated with age and urban district residence. Our results, compared with the previous low prevalence rate (7.7%) detected on Pemba island and the Zanzibar Archipelagos in 2007 (*7*), suggest an endemic pattern of transmission of DENV

Table 2. Multivariable logistic regression model of odds of having IgG against dengue virus, Zanzibar, 2011*

| Covariate† | Adjusted OR (95% CI) | p value |
|---|---|---|
| Age, for 5 y increase‡ | 1.42 (1.27–1.61) | <0.001 |
| Water storage§ | | |
| Yes | 1 | |
| No | 1.66 (0.99–2.76) | 0.053 |
| Area of living | | |
| Rural | 1 | |
| Urban | 4.09 (2.72–6.17) | <0.001 |

*OR, odds ratio.
†Also adjusted for sex, resting water.
‡Age, for 5-y increase is defined as the OR for every 5 years of increase.
§Water storage is defined as any container used to collect rain water with no mention of the size and type of container.

infection in Zanzibar, similar to the situation in other African countries (*10*). Considering the progressive reduction of laboratory-confirmed malaria cases in Zanzibar and the nonspecific influenza-like symptoms of DENV primary infections, this wide DENV circulation in Zanzibar appears to be largely underdiagnosed. Patients with primary DENV infection are likely to be mistakenly treated with antimalarial drugs on the basis of clinical symptoms, as we observed on Pemba (*11*).

We cannot determine when the donors in this study were infected. The strong association with age could be explained by the progressively longer exposure of the older donors to the risk for infection. This association could be also explained by the successful malaria vector control initiative (use of long-lasting insecticidal nets, indoor residual spraying and biolarviciding at mosquito breeding sites, and environmental management), which could account for the lower DENV prevalence in the younger population (*12*). Of note, in our study the proportion of persons who used bed nets and those who did not keep water storage containers near home was quite low.

However, before drawing firm conclusions, a few limitations must be described. First, a high proportion of participants were male. This may have been because of the low proportion of women who donate blood, a consequence of the cultural belief that women are weaker because of blood losses during menstrual periods and pregnancies. This explanation was reported by the workers at ZNBTS and is in accordance with results from a previous study on African immigrants (*13*). Our sample population was thus less representative of the general population and could have affected the results, either by underestimating or overestimating the inferred prevalence. Second, we did not attempt to detect DENV circulating serotypes in this study; nevertheless, there is evidence of DENV-3 circulation in previous reports about imported DENV cases in Europe and Japan (*4*–*6*). Third, no antibody neutralizing assay has been performed to rule out cross-reactions with other circulating flaviviruses, but epidemiologic data from Zanzibar do not indicate outbreaks of other flavivirus infections. Our data constitute the first step toward better defining the circulation of DENV in the Archipelago and toward building up a preparedness and response plan to fight DENV infection.

## Acknowledgments

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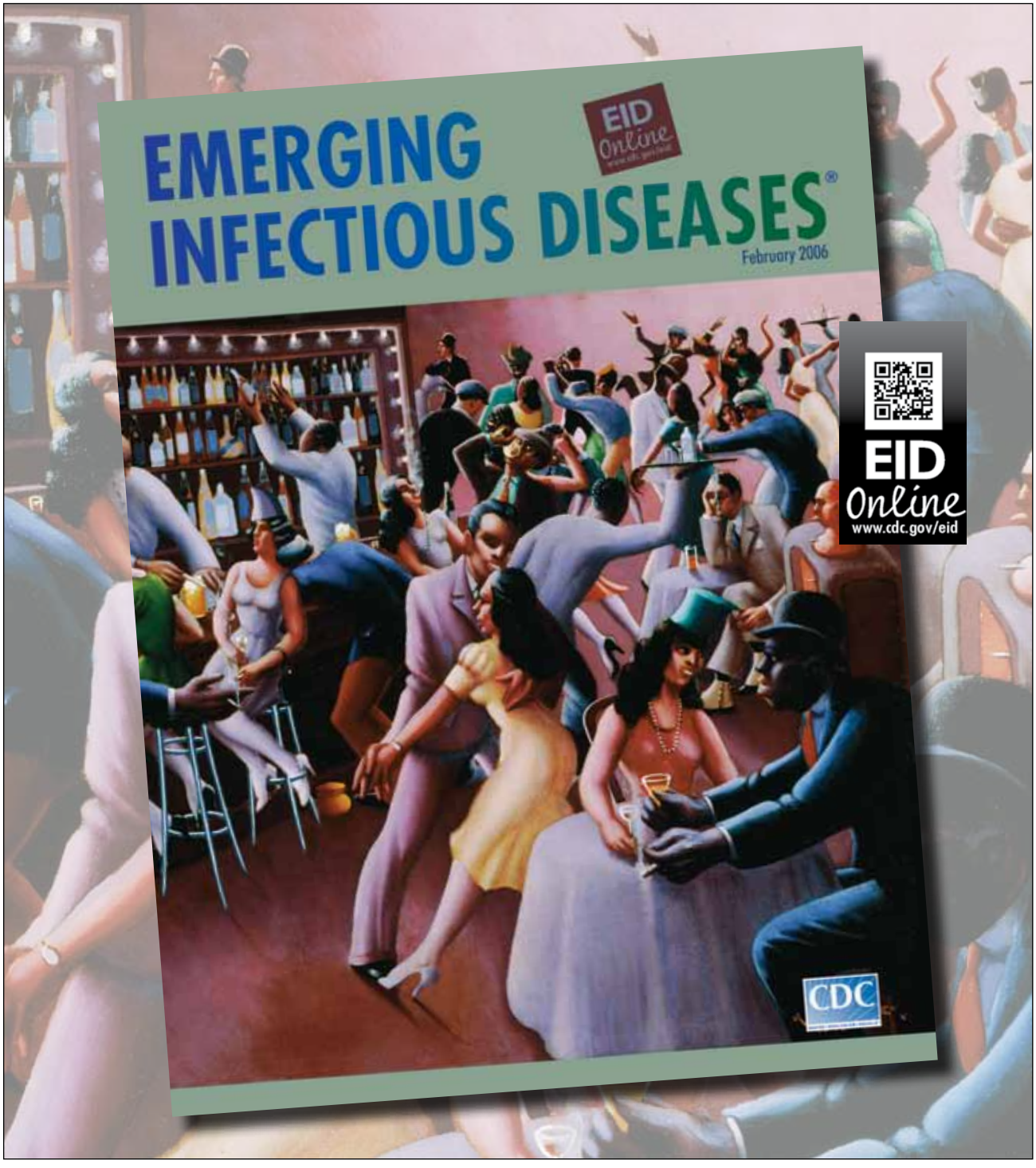

# Mimivirus Circulation among Wild and Domestic Mammals, Amazon Region, Brazil

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To investigate circulation of mimiviruses in the Amazon Region of Brazil, we surveyed 513 serum samples from domestic and wild mammals. Neutralizing antibodies were detected in 15 sample pools, and mimivirus DNA was detected in 9 pools of serum from capuchin monkeys and in 16 pools of serum from cattle.

The group of nucleocytoplasmic large DNA viruses includes viruses that are able to infect different hosts, such as animals, green algae, and unicellular eukaryotes (*1*). Several members of this group are widely distributed in various environments, actively circulate in nature, and are responsible for outbreaks of medical importance (*2*,*3*). *Mimiviridae*, the newest family in this group, has been researched as a putative pneumonia agent and found in different biomes worldwide (*3*,*5*–*9*). The ubiquity of freeliving amebas and their parasitism by mimiviruses enhances the prospect that diverse environments could shelter these giant viruses (*8*–*10*). Mimiviruses can induce infection in a murine model, have had antibodies detected in patients with pneumonia, and can replicate in murine and human phagocytes (*11,12*). Moreover, although some authors suggest that mimivirus is a not frequent pneumonia agent (*4*), mimivirus has been isolated from a human with pneumonia (*3*).

The biomes in Brazil, particularly in the Amazon region, provide the diversity, species richness, and ecologic relationships ideal for identifying circulation of mimiviruses. Preliminary studies found *Acanthamoeba polyphaga mimivirus* (APMV) genomes in samples of bovine serum from Germany (*13*,*14*), indicating that the analysis of samples from vertebrates could be a way to explore and understand the circulation of this group of viruses in nature. We describe the detection of mimivirus antibodies and DNA in 2 mammalian species in the Amazon region of Brazil.

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## The Study

We selected 321 serum samples collected from wild monkeys from the Amazon region of Brazil during 2001–2002: 91 from black howler monkeys (*Alouatta caraya*) and 230 from capuchin monkeys (*Cebus apella*). Samples were collected in an overflow area of a fauna rescue program during the construction of a hydroelectric dam in Tocantins State (Figure 1, Appendix, wwwnc.cdc.gov/EID/article/20/3/13-1050-F1.htm). The monkeys had no previous contact with humans. After blood collection, the animals were released into areas selected by environmental conservation programs. We also collected serum samples from cattle (*Bos taurus*): 147 samples from Pará and Maranhão States in the Amazon region and 45 from Bahia and Espírito Santo States in the Caatinga and Mata Atlântica biomes.

All samples underwent serologic and molecular testing for mimivirus (Figure 2). Because total serum volumes were low, the specimens were grouped into pools of 2–5 serum samples (20 μL for each sample) from animals belonging to the same species that were from the same collection area. A total of 210 pools were compiled (Table). Pools were tested by real-time PCR targeting the conserved helicase viral gene (primers 5′-ACCTGATCCACATCCCATAACTAA-3′ and 5′-GCCTCATCAACAAATGGTTTCT-3′). DNA extractions were performed by using phenol-chloroform-isoamyl alcohol, and DNA quality and concentration were checked by using a nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). PCRs were performed by using the One Step SYBr Green Master Mix (Applied Biosystems, Foster City, CA, USA), and real-time PCR quality and sensitivity parameters were adjusted, including efficiency (102.6%) and $R^2$ (0.992). APMV (kindly provided by Didier Raoult, Marseille, France) was used as a positive control. The serum pools were manipulated in a laminar flow cabinet, separate from any virus samples, to avoid cross-contamination.

Of the 210 pools, 25 (11.9%) were positive for APMV (viral loads $1.4 \times 10^3$ to $2.3 \times 10^6$ copies/mL); 9 (4.3%) pools were capuchin monkey serum and 16 (7.6%) were bovine serum, all from the Amazon region. Mimivirus DNA was not detected in serum from black howler monkeys or cattle from Bahia and Espírito Santo States (Table). Using external primers 5′-ACCTGATCCACATCCCATAACTAAA-3′ and 5′-ATGGCGAACAATATTAAAACTAAAA-3′, we amplified a larger fragment of the helicase gene (390 bp) from all 25 positive samples; 12 positive serum pools, 4 from capuchin

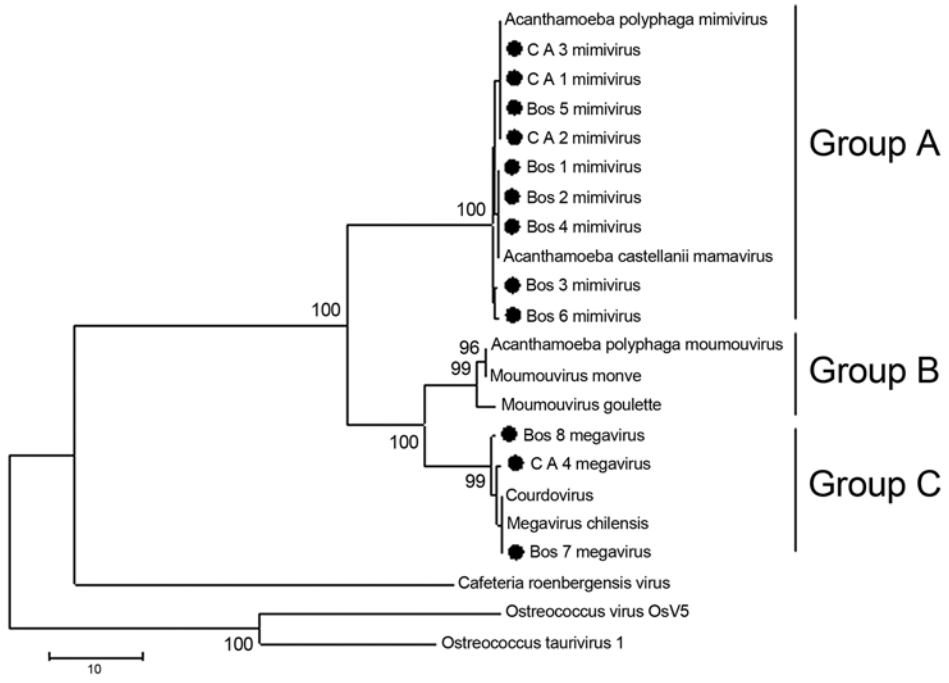


Figure 2. Consensus bootstrap phylogenetic neighbor-joining tree of helicase gene from nucleocytoplasmic large DNA viruses showing alignment of mimivirus and megavirus isolates obtained from *Cebus apella* (CA) and bovids (Bos) in Brazil. Tree was constructed by using MEGA version 4.1 (www.megasoftware.net) on the basis of the nucleotide sequences with 1,000 bootstrap replicates. Bootstrap values >90% are shown. Nucleotide sequences were obtained from GenBank. Scale bar indicates rate of evolution.

monkeys and 8 from cattle, were chosen for helicase gene sequencing and analysis. The helicase fragments were directly sequenced in both orientations and in triplicate (MegaBACE sequencer; GE Healthcare, Buckinghamshire, UK). The sequences were aligned with previously published sequences from GenBank by using ClustalW (www.clustal.org) and manually aligned by using MEGA software version 4.1 (www.megasoftware.net). Modeltest software (www.molecularevolution.org/software/phylogenetics/modeltest) was used determine which model of evolution was most appropriate for our analysis.

Optimal alignment of the predicted highly conserved helicase amino acid sequences showed several amino acid substitutions in the mimivirus amplicons we derived compared with other available sequences (Figure 3). Nine of the 12 sequenced amplicons showed high identity among themselves and with the APMV sequence (GenBank accession no. HQ336222). The other 3 amplicons showed a high identity with *Megavirus chilensis* (GenBank accession no. JN258408), a giant virus isolated in 2011 from seawater off the coast of Chile (*15*). A neighbor-joining phylogenetic tree constructed on the basis of the helicase gene revealed that all the amplicons we derived clustered with mimivirus isolates; however, according to the sequences alignment analysis, 3 of them clustered directly with the *Megavirus chilensis* group (Figure 2). The sequences have been deposited in GenBank.

Concomitantly with molecular analysis, the pools of samples were submitted to a virus neutralization (VN) test to detect mimivirus neutralizing antibodies. VN was used rather than ELISA because the secondary antibodies required for an ELISA for monkey species were unavailable. Before being arranged in pools, the serum samples were inactivated separately by heating at 56°C for 30 min. Inactivated samples were diluted 1:20, mixed with $10^7$ APMV particles to a final volume of 400 μL (peptone yeast glucose medium), then incubated at 37°C for 1 h to optimize virus–antibody interaction. This solution was added to 2 × $10^5$ *Acanthamoeba castellanii* cells (ATCC 30234). To improve virus–ameba contact, the adsorption step was performed while rotating for 60 min. The samples were then centrifuged at 400 × *g* for 5 min, the supernatants were discarded, and the amebas were cultivated at 32°C for 8 h in PYG medium. Afterward, the samples were titrated in *A. castellannii* cells by using the 50% tissue culture

Table. Sources and test results for serum samples from wild and domestic animals analyzed for presence of mimivirus, Brazil*

| Species | States where samples were collected | Total no. samples | Total no. pools | Real-time PCR, helicase gene: No. (%) negative pools | Real-time PCR, helicase gene: No. (%) positive pools | VN >90%, no. (%) pools |
|---|---|---|---|---|---|---|
| Black howler monkeys | Tocantins | 91 | 21 | 21 (100.0) | 0 | 0 |
| Capuchin monkeys | Tocantins | 230 | 106 | 97 (91.5) | 9 (8.5) | 5 (4.72) |
| Cattle | Pará and Maranhão | 147 | 63 | 47 (74.6) | 16 (25.4) | 10 (15.9) |
| | Espírito Santo and Bahia | 45 | 20 | 20 (100.0) | 0 | 0 |
| Total | | 513 | 210 | 185 (88.1) | 25 (11.9) | 15 (7.14) |

*VN, virus neutralization test.

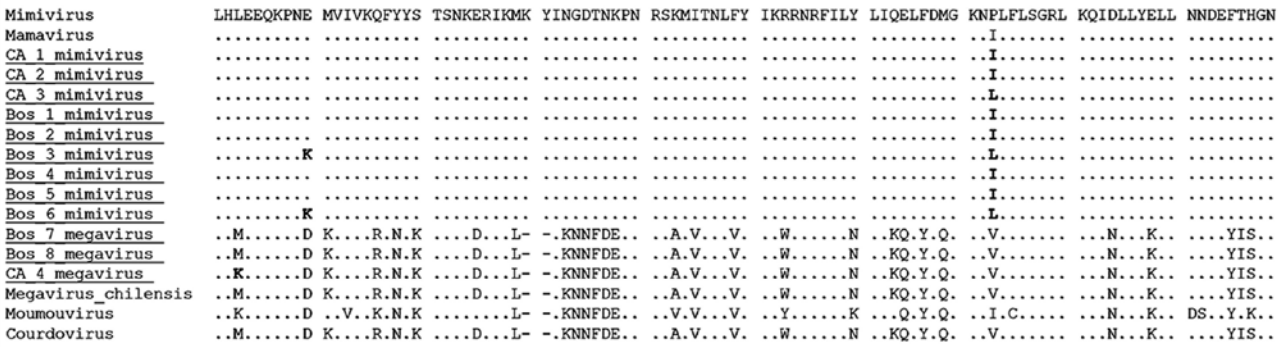


Figure 3. Amino acid inferred sequence of a fragment of the nucleocytoplasmic large DNA virus helicase gene (130 aa were inferred from the obtained 390-bp sample). Samples obtained in this study are underlined; boldface indicates polymorphic.

infective dose method. Antimimivirus serum produced in Balb/c mice was used as VN positive control, and bovine serum collected during previous studies by our group was used as VN negative control. The percentage of reduction was calculated, and the cutoff for positive serum was defined as 90% of the reduction in comparison with the negative control. VN results showed that 15 of the 25 PCR-positive pools contained neutralizing antibodies against mimivirus, 5 from *C. apella* monkeys and 10 from cattle (Table).

## Conclusions

We found evidence of mimivirus circulation in wild and domestic animals in the Amazon region of Brazil. Several agents of emerging infectious diseases in humans have reservoirs in wild and domestic animals, which act as a regular source for these agents. Anthropogenic disturbance of the Amazon ecosystem and increases in agricultural and livestock areas result in more contact between wildlife and rural human populations (*2*). Therefore, although mimivirus-associated pneumonia has not been studied in human patients in Brazil, surveillance of wild and domestic animals can help predict outbreaks and lead to establishment of control measures.

Although mimiviruses are known to be present in water and soil environments, new studies are necessary to determine if these viruses are a part of a vertebrate's normal microbiota and act as opportunistic pathogens for pneumonia and to clarify whether viruses that are associated with pneumonia have any special genetic and physiologic features. Ecologic and public health studies should be designed to evaluate the risk for infection by mimiviruses during wildlife conservation efforts and to determine whether surveillance systems can predict outbreaks by monitoring mimivirus infections in wild and domestic animals.

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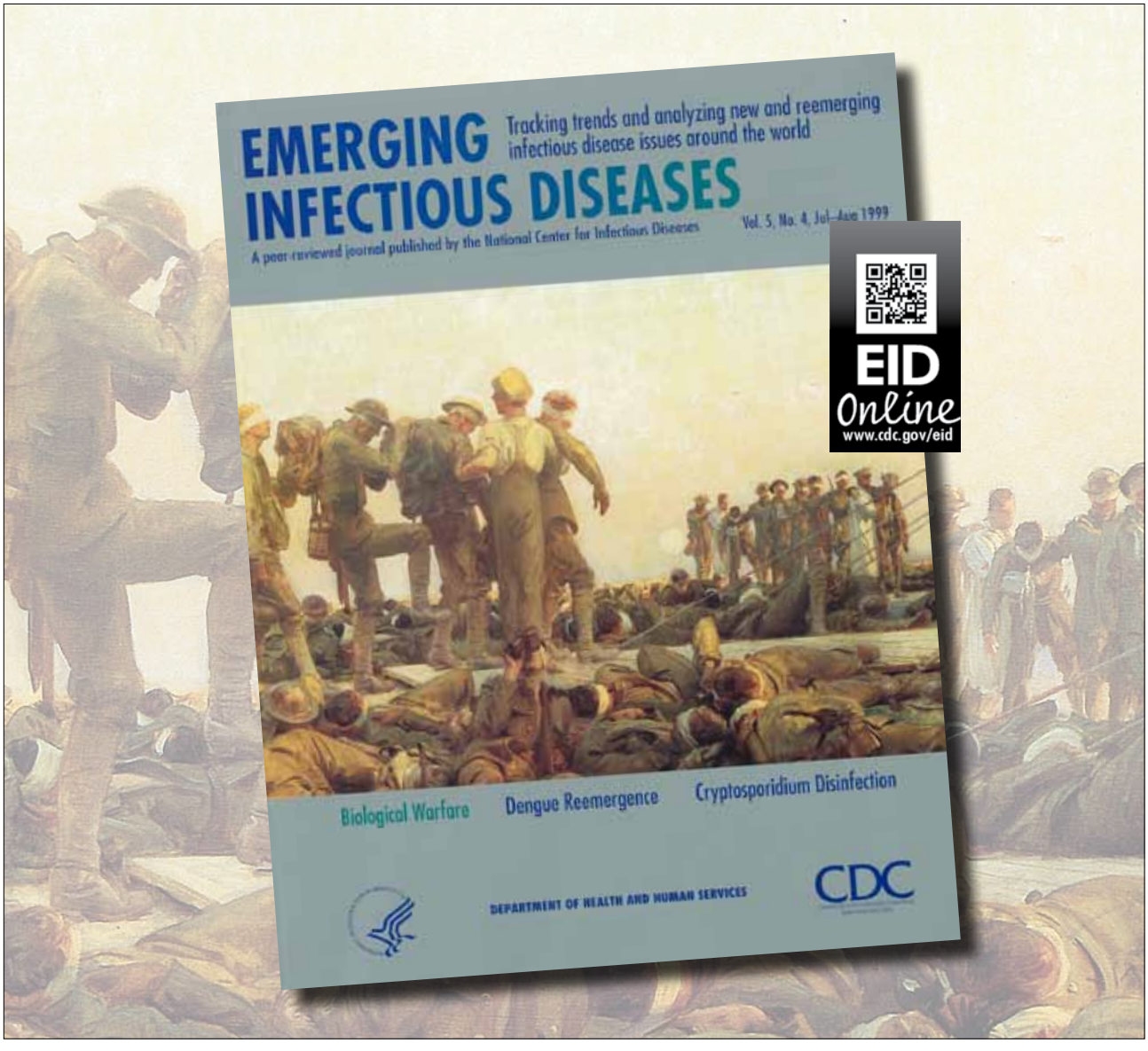

# Infective Endocarditis in Northeastern Thailand

**George Watt, Orathai Pachirat, Henry C. Baggett, Susan A. Maloney, Viraphong Lulitanond, Didier Raoult, Saithip Bhengsri, Somsak Thamthitiwat, Anucha Paupairoj, Michael Kosoy, Nongrak Ud-Ai, Wichuda Sukwicha, Toni Whistler, and Pierre-Edouard Fournier**

Despite rigorous diagnostic testing, the cause of infective endocarditis was identified for just 60 (45.5%) of 132 patients admitted to hospitals in Khon Kaen, Thailand, during January 2010–July 2012. Most pathogens identified were *Viridans streptococci* and zoonotic bacteria species, as found in other resource-limited countries where underlying rheumatic heart disease is common.

Serologic testing of patients with blood culture–negative endocarditis has identified *Coxiella burnetii,* the causative agent of Q fever, and *Bartonella* spp. as noteworthy causes of infective endocarditits (IE) in resource–limited countries (*1*–*4*). Many cases of IE that were not diagnosed by standard blood culture were caused by zoonotic bacteria (*5*). Prospective, systematic descriptions of the etiology and characteristics of IE in Southeast Asia are lacking. We therefore collected detailed clinical, laboratory, and epidemiologic information for patients with confirmed IE in Khon Kaen, Thailand, and conducted specialized testing methods in addition to standard blood cultures to facilitate assessment for zoonotic and nonzoonotic bacteria as the cause of IE.

## The Study

During January 25, 2010–July 19, 2012, patients were prospectively enrolled in this study at 2 tertiary care referral hospitals located on the campus of the medical school of Khon Kaen University. Srinagarind Hospital is a 777-bed general hospital, and the Queen Sirikit Heart Center of the Northeast is a 200-bed specialized cardiac center in which ≈10 heart valve replacement surgeries are performed each month. Patients with suspected IE are referred from much of northeastern Thailand, a region of ≈21 million persons.

Transthoracic echocardiography was performed for patients suspected of having IE; consenting patients ≥16 years of age who met modified Duke criteria for endocarditis were enrolled in this study. Underlying cardiac conditions were assessed by cardiologists on the basis of patients' medical records, history, physical examination, and echocardiographic findings. At admission to a hospital, 3 separate blood samples for culture were obtained in <90 minutes. Blood was inoculated into aerobic medium (BD BACTEC Plus Aerobic/F Medium; Becton Dickinson, Franklin Lakes, NJ, USA), and cultures were processed by using an automated system (BD Bactec Fx series; Becton Dickinson). Pathogens were identified to species level whenever possible, but some blood culture isolates were defined only to the genus level (e.g., viridans group streptococci). One month after admission, a convalescent-phase serum specimen was obtained from each study patient and these patients were evaluated by a cardiologist.

Acute- and convalescent-phase serum specimens were tested for *C. burnetii* and *Legionella pneumophila* by indirect immunofluorescence assay (IFA) as described (*5*). Phase 1 IgG reciprocal titers >800 for *C. burnetii* and total antibody reciprocal titers ≥256 for *L. pneumophila* on either serum specimen were defined as positive (*5*). IFA IgG reciprocal titers of ≥800 to *Bartonella quintana* and *Bartonella henselae* were deemed positive. Specific antibodies to *Brucella melitensis* and *Mycoplasma pneumoniae* were detected with a commercial immunoenzymatic antibody test (*Brucella* antibody and Platellia *M. pneumoniae* IgM kits, respectively; Bio-Rad, Marnes-la-Coquette, France). Reciprocal titers ≥200 were considered positive. DNA was extracted from surgically excised heart valves by using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Courtaboeuf, France) as described by the manufacturer. Previously described broad spectrum PCR primers and amplification and sequencing conditions (*4*) were used to detect all bacteria (16S rRNA); all fungi (18S rRNA); *Staphylococcus aureus*, mitis and gallolyticus group streptococci, *Enterococcus faecalis* and *E. faecium*, *Mycoplasma hominis*, *C. burnetii*, *Bartonella* spp., and *Tropheryma whipplei*.

Table 1 describes characteristics of the 132 enrolled patients: the median age was 47 years (range 16–85) and 68.9% were male. Most of the study patients lived in rural areas, most had a history of animal contact, and most were farmers of rice or vegetables. All study patients had definite IE as determined by using modified Duke criteria.

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Table 1. Characteristics of 132 patients with infective endocarditis, Khon Kaen, Thailand, January 2010–July 2012

| Characteristic | No. patients (%)* |
|---|---|
| Demographic | |
| Median age, y (range) | 47 (16–85) |
| Male sex | 91 (68.9) |
| Live in rural area | 111 (84.1) |
| Occupation | |
| Poultry farmer | 74 (56.1) |
| Livestock farmer | 39 (29.6) |
| Rice farmer | 59 (44.7) |
| Vegetable farmer | 28 (21.2) |
| Housewife | 9 (6.8) |
| Animal contact | |
| Own pets | 92 (69.7) |
| Own livestock | 46 (34.9) |
| Own poultry | 89 (67.4) |
| Underlying cardiac pathologic changes | |
| Identified underlying cardiac condition | 96 (72.7) |
| Rheumatic heart disease | 37 (28.0) |
| Mitral valve prolapse | 19 (14.4) |
| Prosthetic heart valve | 13 (9.9) |
| Congenital heart disease | 11 (8.3) |
| Degenerative valve disease | 9 (6.8) |
| Other cardiac conditions | 4 (3.0) |
| Clinical findings | |
| Type of heart valves involved | |
| Native | 120 (91.1) |
| Prosthetic | 12 (9.9) |
| Heart valves involved overall, n = 132 | |
| Aortic | 62 (47.0) |
| Mitral | 52 (39.4) |
| Tricuspid | 2 (0.2) |
| Aortic and mitral | 10 (7.6) |
| Mitral and pulmonary | 1 (0.01) |
| Aortic and tricuspid | 1 (0.01) |
| Mitral and tricuspid | 1 (0.01) |
| Aortic, mitral, and tricuspid | 1 (0.01) |
| Heart valves involved in patients with underlying rheumatic heart disease, n = 37 | |
| Aortic | 21 (56.8) |
| Mitral | 10 (27.0) |
| Mitral and aortic | 5 (13.5) |
| Mitral, aortic, and tricuspid | 1 (2.7) |
| Days of fever before admission, median (range) | 21 (0–270) |
| Patients receiving antibacterial drugs during week before admission | 77 (58.3) |
| Mean ± SD maximum temperature during first 24 h after admission | 38.3°C.± 0.9°C |
| Congestive heart failure at admission† | 64 (48.5) |

*Data indicate no. (%) patients with specified characteristic unless otherwise indicated.
†Diagnosis was made by a cardiologist.

Patients had high fever and were severely ill at admission; more than half had congestive heart failure (Table 1). Underlying cardiac pathologic changes were identified in 96 (72.7%) study patients (Table 1); rheumatic heart disease (RHD) was the most common condition, as has been found in other resource-limited countries (*1*,*2*,*6*,*7*). RHD was identified in 37 (28.0%) of the 132 patients overall; the 37 represented 38.5% of the 96 patients with identified underlying cardiac pathologic changes. IE was detected on prosthetic heart valves in 12 (9.9%) of the study patients, including 2 patients whose blood cultures grew coagulase-negative staphylococci.

Of the 132 study patients, 100 (75.8%) underwent surgery. Such management reflects current international guidelines for complicated IE, which emphasize identifying high-risk patients, transferring them to a specialized medical–surgical center, and performing early valvular surgery (*8*). Within 1 month, 11 (8.3%) of the 132 patients died and 6 were still hospitalized; 5 died after discharge. Four additional patients are known to have died after leaving the study, but we did not systematically assess survival beyond the 1-month follow-up examination. The case-fatality proportion calculated on the basis of known deaths was 11.4%, but the possibility of additional fatalities cannot be excluded. The mortality rate was 18.8% for patients who did not undergo surgery and 9.0% for those who did.

A pathogen was identified for 60 (45.5%) cases (Table 2). The etiologic agent was identified for 7 (21.9%) of 32 nonsurgical cases, 5 by blood culture and 2 by IFA,

compared with 53 (53.0%) of the 100 study patients who underwent surgery (Fisher exact test, p = 0.02). Among patients who underwent surgery, diagnosis was made by PCR of heart valve tissue for 29, by blood culture for 11, and by >1 method for the remaining 13; no discrepancies between results from different diagnostic modalities were found. Many (80.3%) patients had been referred from other hospitals for management of IE and were already being treated for that illness. We confirmed that 77 (58.3%) patients had received antibacterial drugs during the week before admission. We suppose that the low diagnostic yield was associated with antibacterial drug use, but more detailed information is needed to determine the accuracy of this supposition.

Among the 60 cases of IE for which a causative pathogen was identified, 15 (25.0%) were attributed to zoonotic bacteria (Table 2): *C. burnetti* (5 cases), *B. henselae* (4 cases), *Streptococcus suis* (4 cases), *Erysipelothrix rhusiopathiae* (1 case), and *Campylobacter fetus* (1 case). *B. quintana* causes ≈75% of *Bartonella* IE cases worldwide (*9*) but was not found in this study. Verification that *C. burnetti* (*10*), *Bartonella spp.* (*11*), *E. rhusiopathiae*, and *C. fetus* cause IE in Thailand highlight the noteworthiness of emerging zoonotic pathogens in this region. The identification of Q fever IE stimulated efforts by public health authorities of Thailand to characterize Q fever prevalence and transmission nationwide.

Streptococci generally cause the majority of infections of native heart valves (*12*), and 90.1% of patients in this study had native valve involvement. Viridans streptococci, including *S. suis*, accounted for 43.3% of cases of known etiology (Table 2) and included 3 species of streptococci not previously reported to cause IE in Thailand (*S. gallolyticus*, *S. difficilis*, and *S. sinensis*). In Thailand, as elsewhere, health care–associated IE and IE among intravenous drug users is often caused by *S. aureus* (*13*,*14*). None of the patients in this study were known to be intravenous drug users, and few had health care–associated infections; 5 (8.3%) of 60 cases were caused by *S. aureus*.

Table 2. Bacterial species identified in 60 patients with infective endocarditis, Khon Kaen, Thailand, January 2010–July 2012*

| Disease type, organisms | No. (%) cases |
|---|---|
| Zoonoses | 15 (25) |
| *Coxiella burnetti*† | 5 |
| *Bartonella henselae*† | 4 |
| *Streptococcus suis* | 4 |
| *Erysipelothrix rhusiopathiae*† | 1 |
| *Campylobacter fetus*† | 1 |
| Nonzoonoses | 45 (75) |
| *Enterococcus faecalis* | 6 |
| *Enterococcus* spp. | 4 |
| *Staphylococcus aureus* | 5 |
| Coagulase–negative *Staphylococcus* sp. | 2 |
| Viridans streptococci | 5 |
| *Streptococcus agalactiae* | 4 |
| *Streptococcus anginosus* | 4 |
| *Streptococcus gordonii* | 2 |
| *Streptococcus gallolyticus*† | 2 |
| *Streptococcus mitis* | 2 |
| *Streptococcus dysgalactiae* | 1 |
| *Streptococcus oralis* | 1 |
| *Streptococcus difficilis*† | 1 |
| *Streptococcus pneumonia* | 1 |
| *Streptococcus sinensis*† | 1 |
| *Streptococcus* spp. | 1 |
| *Corynebacterium diptheriae* | 1 |

*No pathogenic agents were identified for 72 (54.6%) of 132 patients.
†Not previously reported to cause infective endocarditis in Thailand.

## Conclusions

Zoonotic bacteria were detected by specialized testing of 11.4% of IE patients in northeastern Thailand, but an etiology could not be determined for more than half (54.5%) of the patients. Infections with viridans streptococci predominated among cases of known etiology, and RHD was the most common underlying pathologic change. To increase diagnostic yield, we recommend intensification of efforts to obtain blood cultures in the absence of antimicrobial drugs and increase of laboratory capacity to test for zoonotic bacteria (*15*).

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# Crimean-Congo Hemorrhagic Fever among Health Care Workers, Turkey

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We investigated 9 cases of Crimean-Congo hemorrhagic fever (1 fatal, 2 asymptomatic) among health care workers in Turkey. Needlestick injuries were reported for 4 workers. Eight received ribavirin. In addition to standard precautions, airborne infection isolation precautions are essential during aerosol-generating procedures. For postexposure prophylaxis and therapy, ribavirin should be given.

Crimean-Congo hemorrhagic fever (CCHF) has been described from Africa, Asia, southeastern Europe, and the Middle East (*1*). The CCHF virus (CCHFV) belongs to the family *Bunyaviridae*, genus *Nairovirus*, and causes severe disease in humans; the reported case-fatality rate is 3%–30% (*1*). Humans become infected through the bites of ticks, contact with infected patients' body fluids, or contact with blood or tissues from viremic livestock. Health care workers (HCWs) are at occupational risk for CCHFV infection. Health care–related CCHFV infections have been reported in Pakistan (*2*–*5*), the United Arab Emirates (*6*), South Africa (*7*), Iran (*8*), India (*9*), Tajikistan (*10*), and Turkey (*11*). We describe the outcomes of 9 HCWs in Turkey who had occupational exposure to CCHFV.

## The Cases

The 9 HCWs and all CCHF patients under their care were admitted to the Infectious Diseases and Clinical Microbiology clinic (IDCM) of Ankara Numune Education and Research Hospital (Ankara, Turkey) during 2004–2011 with confirmed CCHF. All 9 HCWs were aware of possible or confirmed CCHFV infection in their patients. During this period, ≈7,000 confirmed CCHF cases were recorded in Turkey; nearly 300 of these patients were hospitalized in IDCM. Acute- and convalescent-phase serum samples from the index patients were sent to the national reference laboratory of Turkey. CCHFV infection was confirmed through IgM positivity by ELISA and/or positive PCR results for CCHFV in blood. After episode 1, the HCWs' serum samples were sent to the Pasteur Institute (Lyon, France) for contact tracing. The HCWs' infections were scored according to a severity score index (*12*). In episode 3, to investigate the source of infection, molecular techniques were used. Oral ribavirin for treatment was administered at the dosage recommended by the World Health Organization (4 g/d for 4 d, 2.4 g/d for 6 d), and for prophylaxis (2g/4×/d for 7 d). The index patients and the HCWs were given erythrocyte, fresh frozen plasma, and total blood preparations depending on their homeostasis.

### Episode 1

In 2005, CCHFV infection was diagnosed in a woman on the day of delivery by cesarean section. She was transferred to IDCM, and her baby was transferred to the newborn service at the Dr. Sami Ulus Children's Hospital (Ankara, Turkey). A nurse in IDCM, who had fever and myalgia, was later found to have CCHFV infection (Table 1). Transmission was related to the improper use of gloves during the care of the mother's surgical wound. The mother recovered, but the infant died 5 days after birth. A nurse in the neonatal clinic in the children's hospital also acquired CCHFV infection, which was attributed to the intubation and aspiration of bloody secretions from the baby without proper use of gloves and mask. For both HCWs, the incubation period was 2 days. The first HCW was given ribavirin at symptom onset. The second nurse's illness was mild; because she had a potential for getting pregnant, ribavirin was not started. Both nurses recovered completely (Table 2).

After this episode, the index patient's contacts were traced. Serum samples from 37 HCWs at IDCM and the obstetrics and newborn clinics at the children's hospital who were at risk for infection were investigated for CCHF. In addition to the 2 nurses, 2 nurses from the neonatal clinic were CCHF IgM positive but were asymptomatic.

### Episode 2

In 2006, a nurse received a needlestick injury during a phlebotomy of a CCHFV-infected patient. She was using gloves but no gown, and the needle stuck to her forearm. The infected patient died. The nurse's symptoms began 2 days after the incident, and she was transferred to IDCM 4 days after the incident. At admission, her severity score index was high. She had ecchymosis, epistaxis, hematemesis, melena, vaginal bleeding, and somnolence (Table 1). She received oral ribavirin 5 days after the incident, which possibly had limited effect because her illness already had progressed to confusion and gastrointestinal bleeding. She died on the second day after hospital admission.

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Table 1. Clinical and laboratory findings of HCWs in whom Crimean-Congo hemorrhagic fever developed after occupational exposure, Turkey, 2004–2011*†

| HCW, outcome | Body temperature, °C | Bleeding | Leukocytes/ $mm^3$ | Platelets/ $mm^3$ | AST | ALT | APTT | Fibrinogen | SSI |
|---|---|---|---|---|---|---|---|---|---|
| 1, survived | 38.5 | No | 800 | 42,000 | 425 | 346 | 44 | 225 | Moderate |
| 2, survived | 37.2 | No | 1100 | 53,000 | 145 | 81 | 43 | 270 | Mild |
| 3, died | 40.5 | Ecchymosis, hematemesis, melena, hematuria | 11,100 | 40,000 | 251 | 277 | 90 | 171 | Severe |
| 4, survived | 40.5 | No | 2,900 | 78,000 | 150 | 110 | 37.4 | 250 | Mild |
| 5, survived | 39 | Epistaxis | 1,800 | 58,000 | 167 | 129 | 64 | 218 | Moderate |
| 6, survived | 40.5 | No | 1,800 | 44,000 | 123 | 216 | 40.5 | 165 | Moderate |
| 7, survived | 39.1 | No | 3,100 | 13,000 | 418 | 132 | 40.9 | 170 | Moderate |

*HCW, health care worker; AST, aspartate aminotransferase; ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; SSI, severity score index.
†Reference values: leukocytes, 4,000–11,000/$mm^3$; platelets, 150,000–450,000/$mm^3$; AST, <50 IU/L; ALT, <50 IU/L; APTT, 24–36 sec; fibrinogen, 200–400 mg/dL.

### Episode 3

In 2008, a patient with CCHFV infection and a high viral load ($10^8$ copies/mL) was hospitalized with hemoptysis, hematemesis, melena, epistaxis, and intraalveolar bleeding. He died 3 days after admission. One infectious diseases resident and 2 otorhinolaryngology residents acquired CCHFV infection. All HCWs had worn personal protective equipment during the intervention. One otorhinolaryngology resident performed nasal tamponade. After the intervention, the other otorhinolaryngology resident handled and cleaned a head mirror without using gloves, although he was not in face-to-face contact with the patient. The infectious diseases resident resuscitated the patient without apparent direct contact with the patient's bloody secretions. For the 3 residents, fever, malaise, and myalgia developed 2–5 days after exposure. Ribavirin was started at symptom onset. No contact was observed between the patient and the HCWs, but the RNA sequences from the patients and HCWs were identical by molecular techniques. All of the HCWs recovered.

The transmission of CCHFV could have resulted from indirect contact with contaminated devices, such as the head mirror; the improper removal of gowns, masks, gloves; inadequate hand hygiene; or failure to use N95 masks during aerosolizing procedures. During the procedures that could generate aerosols, HCWs should wear an N95 mask or FFP2 respirator (*13*). The patients with higher viral load were reported to be more severe disease (*14*).

### Episode 4

In 2008, a phlebotomist working in a children's hospital had a needlestick injury during phlebotomy of a CCHFV-infected child. He was hospitalized 1 day after symptom onset, and ribavirin was started. His severity score index was moderate, and he recovered.

Table 2. Demographic features of HCWs with occupational exposure to Crimean-Conger hemorrhagic fever virus, Turkey, 2004–2011*

| Episode, outcome† | HCW age, y/sex/profession | Procedure | Transmission route | Ribavirin for postexposure prophylaxis | Ribavirin for therapy (d after symptom onset) | Fatal |
|---|---|---|---|---|---|---|
| Episode 1; survived, her baby died | 36/M/nurse | Wound care | Contact with surgical wound without protective equipment | No | Yes (0) | No |
| | 31/F/nurse | Intubation, aspiration | Aerosol and droplet and contact without protective equipment | No | No | No |
| Episode 2; died | 28/F/nurse | Phlebotomy | Needlestick | No | Yes (3) | Yes |
| Episode 3; died | 41/M/physician | Resuscitation | Aerosol and droplet | – | Yes (0) | No |
| | 26/M/physician | Nasal tamponade | Indirect contact | – | Yes (0) | No |
| | 29/M/physician | Nasal tamponade | Indirect contact | – | Yes (0) | No |
| Episode 4; survived | 30/M/nurse | Phlebotomy | Needlestick | No | Yes (1) | No |
| Episode 5; survived | 30/F/nurse | Phlebotomy | Needlestick | Yes | – | No |
| Episode 6; survived | 24/F/physician | Phlebotomy | Needlestick | Yes | – | No |

*HCW, health care worker; –, ribavirin not necessary.
†Outcome for the index case-patient in each episode.

### Episodes 5 and 6

A nurse in 2007 and a pediatric resident in 2008 incurred needlestick injuries during phlebotomy of a CCHFV-infected patient. Postexposure prophylaxis with ribavirin was started immediately after the injuries, and no infections developed.

## Conclusions

Six of the 9 CCHF-infected HCWs reported here had histories of needlestick injuries or contact with contaminated blood without adequate barrier precautions. An integrated strategy for controlling accidental exposure to body fluids was developed to protect HCWs against CCHFV infection. All personnel, including cleaning staff in health care units on all shifts were informed and trained about the transmission risks, protection, and clinical symptoms of CCHF (*13*). The standard, contact, and droplet precautions were usually sufficient to protect against CCHFV infection during the routine care of CCHF patients. In addition to the practices of previous years, the airborne infection isolation precautions during aerosol-generating procedures were performed. The number of HCWs caring for patients with severe CCHF was limited. After all of these measures were enforced, occupational CCHFV infection did not occur in IDCM.

Ribavirin is an effective treatment for CCHFV infection (*12*) and beneficial for postexposure prophylaxis (*13*,*15*). Therapy should be started as early as possible. In the 2 HCWs reported here who received ribavirin for postexposure prophylaxis, no symptoms developed; similar reports will be useful for increasing the power of this conclusion. We used a ribavirin dosage of 2g/day, but no consensus exists about the dosage for postexposure prophylaxis.

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# Influenza A(H1N1) pdm09 Virus Infection in Giant Pandas, China

**Desheng Li, Ling Zhu, Hengmin Cui, Shanshan Ling, Shengtao Fan, Zhijun Yu, Yuancheng Zhou, Tiecheng Wang, Jun Qian, Xianzhu Xia, Zhiwen Xu, Yuwei Gao,[1] and Chengdong Wang[1]**

We confirmed infection with influenza A(H1N1)pdm09 in giant pandas in China during 2009 by using virus isolation and serologic analysis methods. This finding extends the host range of influenza viruses and indicates a need for increased surveillance for and control of influenza viruses among giant pandas.

In April 2009, the Centers for Disease Control and Prevention reported the emergence of a novel strain of influenza A(H1N1) virus, which is now referred to as influenza A(H1N1)pdm09 or pH1N1. This virus rapidly affected countries worldwide and continues to circulate as a seasonal influenza virus. In addition to widespread infection of humans, reported have been published of pH1N1 virus infection in domestic and nondomestic animals, including cats, dogs, ferrets, swine, and several wildlife species (*1*–*4*). Here, we report a confirmed case of pH1N1 virus infection in giant pandas (*Ailuropoda melanoleuca*) in China.

## The Study

During the human outbreak of pH1N1 in China, 3 giant pandas at the Conservation and Research Center for the Giant Panda in Ya'an City, Sichuan Province, showed clinical signs suggestive of a respiratory tract infection, including pyrexia, anorexia, malaise, conjunctivitis, and sneezing. Nasal swab specimens were successfully collected under anesthesia from 1 of the affected pandas (Ximeng). All 3 pandas recovered after receiving 75 mg of oseltamivir phosphate twice daily for 5–6 days. For serologic analysis, serum samples were collected from all 3 pandas ≈3 months after the resolution of the respiratory illness; a serum sample collected before onset of the respiratory illness was also available for all 3 animals.

The nasal swab specimens were collected in 1 mL phosphate-buffered saline and tested for evidence of pH1N1 virus and several other pathogens reported (*5*–*7*) or suspected to occur in giant pandas: canine distemper virus, canine adenovirus, canine coronavirus, canine herpesvirus, and canine parainfluenza virus. Testing for detection of influenza A virus was performed by using a real-time reverse transcription PCR method, as described by the World Health Organization (WHO) (*8*); other pathogens were tested by different PCR methods.

RNA from the swab specimens tested positive for the hemagglutinin gene of pH1N1 virus. No other pathogens were detected.

To isolate and characterize the pH1N1 virus, we injected 10-day-old specific pathogen free embryonated chicken eggs with material collected from one of the nasal swab samples. Allantoic fluid from the injected eggs agglutinated 0.5% (vol/vol) chicken erythrocytes, indicating the presence of replication-competent virus in the nasal swab sample. When evaluated by electron microscopy, allantoic fluid supernatant from the infected egg displayed enveloped influenza virus–like particles of 100–120 nm (Figure 1).

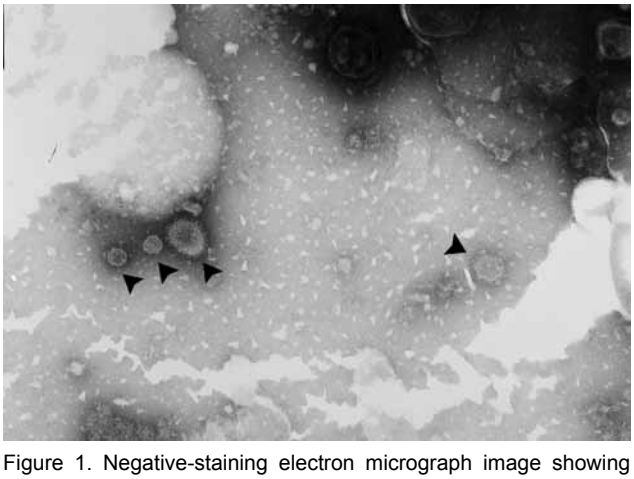

Figure 1. Negative-staining electron micrograph image showing influenza A(H1N1)pdm09 virus particles (arrowheads) in allantoic fluid supernatant collected from specific pathogen free eggs after injection with a nasal swab sample collected from a giant panda in China. Original magnification ×40,000.

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[1]These authors contributed equally to this article.

We sequenced the entire genome of the virus by using RNA harvested from the allantoic fluid of infected eggs. Sequence analysis was performed as described by WHO by using methods for pH1N1 virus (*9*). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify sequences similar to those of the giant panda isolate; these sequences were GenBank accession nos. KF277197–KF277204. Analyses showed that each of the 8 gene segments of the virus we isolated were closely related to pH1N1 viruses circulating among humans, including a human representative strain (A/California/04/2009) and a contemporary strain (A/Sichuan/1/2009); these viruses showed 98.6%–100% nt identity to the panda strain (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/3/13-1531-Techapp1.pdf). Phylogenetic analysis of the 8 gene segments of the virus we isolated showed that the isolate was related to the pH1N1 virus clade of influenza viruses (Figure 2; online Technical Appendix Figure). The results of the BLAST and phylogenetic analyses suggest that a human pH1N1 virus was transmitted directly to giant pandas without recombination or significant adaptation. We named the virus that we isolated A/giant panda/01/Ya'an/2009 (H1N1).

A/giant panda/01/Ya'an/2009 (H1N1) virus contained glutamine at hemagglutinin amino acid position 226 (H3 numbering), which has been found in human pH1N1 isolates and confers binding to human cell-surface receptors (*10*). In addition, alanine at position 271 of polymerase protein 2 of the isolate reported here has been shown to be required for respiratory droplet–mediated transmission of A/Sichuan/01/2009 virus (*10*). Thus, the genomic sequences of the giant panda isolate appear to have many features

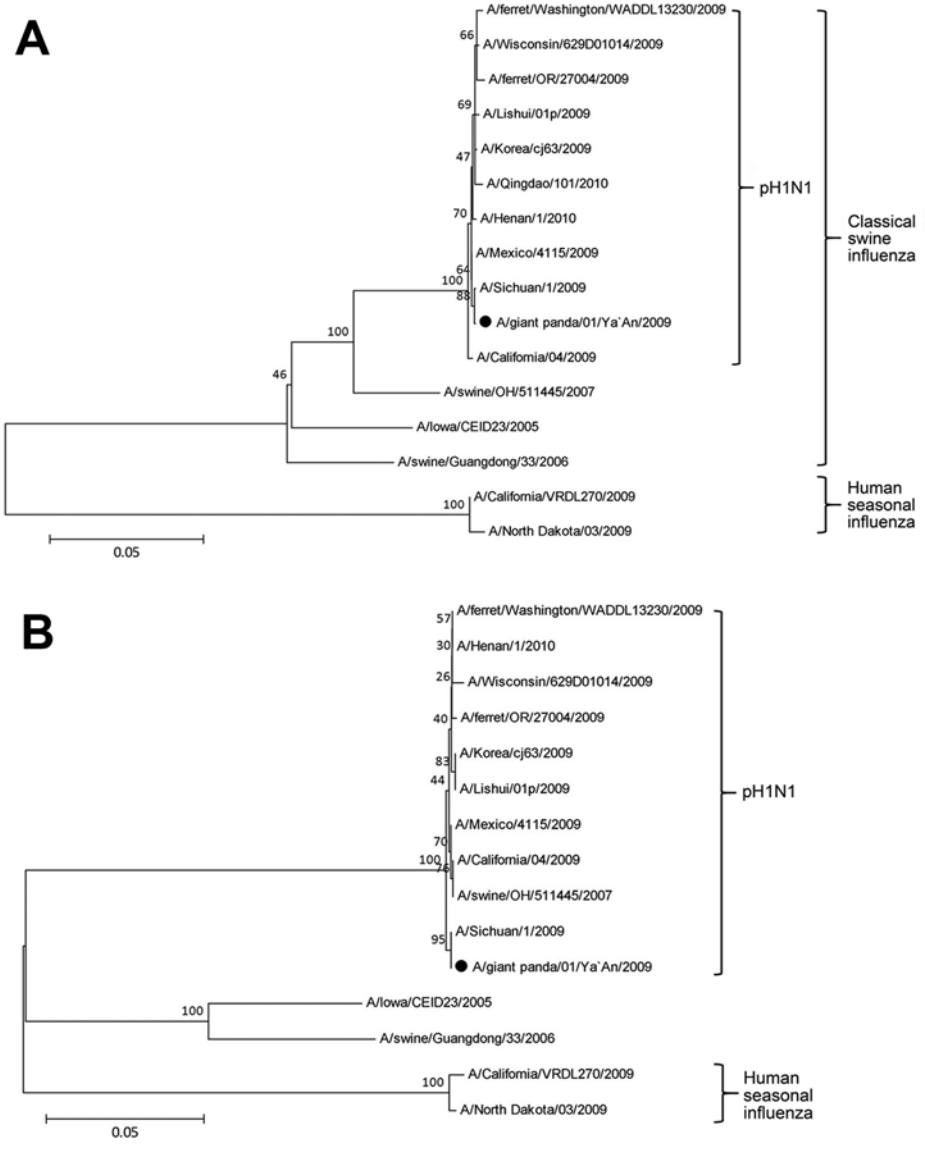


Figure 2. Phylogenetic trees of influenza A(H1N1)pdm09 virus (pH1N1) isolated from a giant panda in China compared with previously characterized pandemic influenza A(H1N1) viruses. A) Hemagglutinin gene nucleotide sequences; B) neuraminidase gene nucleotide sequences. Neighbor-joining trees were created by using MegAlign software version 5.0 (www.megasoftware.net). Bootstrapping with 1,000 replicates was performed to determine the percentage reliability for each internal node. Horizontal branch lengths are proportional to genetic distances. Black dot indicates the isolate from this study. Scale bars indicate nucleotide substitutions per site.

Table. Hemagglutination inhibition antibody titers against influenza A viruses in serum samples collected from giant pandas before and after respiratory infection, China, 2009*

| Time | Animal name | Date collected | Titer | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | A(H1N1)pdm09 | H3 | H6 | H5 | H7 | H9 |
| Before infection | Ximeng | 2004 Oct 5 | <10 | ND | ND | <10 | ND | ND |
| | Gege | 2009 Jul 29 | <10 | <10 | <10 | <10 | <10 | <10 |
| | Zhangka | 2009 Dec 1 | 320 | 80 | <10 | <10 | <10 | <10 |
| After infection | Ximeng | 2010 Mar 23 | 640 | <10 | <10 | <10 | <10 | <10 |
| | Gege | 2010 Mar 31 | 640 | <10 | 80 | <10 | <10 | <10 |
| | Zhangka | 2010 Mar 27 | 640 | 80 | 40 | <10 | <10 | <10 |

*All serum samples were pretreated with *Vibrio cholerae* receptor–destroying enzyme (Sigma-Aldrich, St. Louis, MO, USA) before testing. ND, not done.

known to be important for the replication and transmission of pH1N1 viruses in other mammalian species.

Serum samples collected from the affected pandas before their illnesses and 3 months after the resolution of the illnesses were tested for the presence of hemagglutination inhibition (HI) antibodies against a panel of influenza A viruses. HI antibody titers were measured against the A/giant panda/01/Ya'an/2009 (H1N1) isolate, human seasonal influenza (H3N2) virus (A/Victoria/361/2011), avian (H5N1) virus (A/duck/Anhui/1/2006), avian (H6N1) virus (A/Mallard/SanJiang/275/2007), and avian (H7N1) virus (A/Baer's pochard/HuNan/414/2010). All 3 pandas had high titers of HI antibodies against the pH1N1 virus 3 months after illness (Table).

One panda (Zhangka) had detectable pH1N1 and H3 subtype HI antibodies before infection; this serum sample was collected before the display of overt clinical signs in the animal but after the onset of the human pH1N1 outbreak in China. It is unknown if these antibodies reflect previous exposure to pH1N1 virus or are reflective of cross-reactive antibodies generated against antigenic sites in previously circulating influenza viruses.

Two of the pandas (Gege and Zhangka) also had serum antibodies that inhibited hemagglutination mediated by an H6 subtype avian influenza virus 3 months after the respiratory infection, whereas no such antibodies were detected before the respiratory infection (Table). When reference serum samples known to contain HI antibodies against each of the viral subtypes were evaluated for potential cross-reactivity against the other influenza subtypes, we observed no apparent cross-reactivity of pH1N1 antibodies against the H6 subtype virus (online Technical Appendix Table 2). Therefore, although it is possible that the high titers of HI antibodies against pH1N1 virus in these animals cross-reacted with the H6 subtype viruses, we cannot exclude the possibility that these antibodies were generated in response to an independent exposure to an H6 influenza virus.

## Conclusions

Influenza A(H1N1)pdm09 virus infection has been found in mammals and birds of a variety of species since the virus was identified in early 2009 (*1–4*). We documented infection with this virus in giant pandas in China during 2009. Sequence analysis of a virus isolated from a nasal swab sample revealed that it was nearly identical to the pH1N1 virus circulating in humans, which suggests that the virus may have been transmitted to the pandas directly from humans. Furthermore, serologic analysis of samples from the affected pandas was consistent with productive infection by pH1N1 virus. Seroconversion was documented in each animal, although 1 animal appeared to have been previously exposed to pH1N1 or a related virus that was able to elicit cross-reactive antibodies. Similarly, some pandas had detectable HI antibodies against H3 and H6 subtype influenza viruses, raising the possibility that giant pandas may be infected with human- and avian-origin influenza viruses.

Only 1,600 wild and 300 captive giant pandas remain worldwide (*11*). Our findings highlight the risk posed by influenza viruses to these animals, extend the known host range of influenza A viruses, and have implications for wildlife conservation and influenza virus epidemiology. When taken together with previous reports of pH1N1 infection of other mammals, our data suggest that this virus can efficiently infect a wider range of mammalian species than can other influenza viruses. Increased surveillance and control of influenza viruses in giant panda populations are imperative for successful conservation of these endangered animals.

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# Mixed Scrub Typhus Genotype, Shandong, China, 2011

**To the Editor:** Scrub typhus, which is caused by the bacterium *Orientia tsutsugamushi*, had been considered a disease of the tropical zone in China until the first outbreak in the temperate zone of Shandong Province in 1986 (*1*). The Sdu-2 genotype had not been reported for human patients in China since its detection in the striped field mouse (*Apodemus agrarius*) during 2007 (*2*). We report a case in a person in Shangdong Province who was co-infected with the HSX and Sdu-2 genotypes of *O. tsutsugamushi.*

A female farmer, 55 years of age, was admitted to Mengyin County Hospital on September 29, 2011, with a 5-day history of fever (highest temperature 39°C), headache, back pain, chills, anorexia, and nausea. Her back pain was exacerbated as her fever increased. A black eschar (10 mm × 20 mm) was observed above the left breast, and 2 enlarged lymph nodes (20 mm × 30 mm) that were tender to the touch were found behind the right ear. The patient's Glasgow Coma Scale score was 15. No comorbidities were reported. Laboratory tests showed slight leukopenia, low potassium level, decreased albumin/globulin ratio, elevated lactate dehydrogenase level, and elevated C-reactive protein level. Results of urine dry chemical analysis were positive for occult blood and leukocytes. The Weil-Felix reaction to the *Proteus* OXK antigen was negative. Mild abnormalities were observed in results of an electroencephalogram and an electroencephalogram topographic map. The patient was suspected to have scrub typhus complicated by encephalitis and urinary tract infection. Doxycycline (200 mg once daily) and symptomatic treatment were administered immediately. The patient's symptoms had generally subsided on day 5 of hospitalization. Indirect immunofluorescence assay showed a 4-fold rise in IgG titer against *O. tsutsugamushi* between acute-phase (128) and convalescent-phase (512) serum samples collected 18 days apart.

Acute-phase whole blood was collected before administration of antimicrobial drugs. The eschar was collected after natural desquamation. DNA from the whole blood and eschar was isolated and screened for *O. tsutsugamushi* by using PCR primers E and B to target the 56-kDa type-specific antigen gene (*3*). Type-specific amplification of *O. tsutsugamushi* was performed (*4*,*5*). Normal values for fragment yield are 407, 230, 242, 220, 600, and 523 bp for Gilliam, Karp, Kato, Kuroki, Saitama, and Kawasaki genotypes, respectively.

Three products that had the same molecular weight as those from Kato, Saitama, and Kawasaki genotypes were generated from the blood sample by using type-specific amplification. However, sequence analysis of amplicons yielded by using type-specific primers and those yielded by using primers E and B demonstrated a co-infection in the blood with 2 genotypes of *O. tsutsugamushi*, which were designated as ZZF-KW (661 bp, GenBank accession no. JX644590) and ZZF-HSB (498 bp, GenBank accession no. JX644591). Co-infection with the 2 genotypes was also detected in the eschar. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis showed that ZZF-KW had 100% sequence similarity to the HSX genotype of *O. tsutsugamushi* (657 bp, GenBank accession no. JX202566) and that ZZF-HSB had 100% sequence similarity to Sdu-2 genotype (476 bp, GenBank accession no. EF543196). A nucleotide identity of 72% was shown between ZZF-KW and ZZF-HSB. Nucleotide identities of partial 56-kDa type-specific antigen genes among the 2 *O. tsutsugamushi* genotypes and the reference types are shown in the Table.

Co-infection with HSX and Sdu-2 genotypes of *O. tsutsugamushi* was detected in a single case. Mixed genotype infections could not be detected by using 1 amplicon, which could amplify only a predominant genotype. Type-specific amplification is a convenient method for the detection of mixed genotype infection, although this method has limitations for identification of novel genotypes. The Sdu-2 genotype was not differentiated from Kato, Saitama, and Kawasaki genotypes by using type-specific primers, but differentiation was achieved by gene sequencing. Considering the genotypic diversity (*6*) and mixed genotype infection of the agent, type-specific primers require redesign for type designation and subsequent sequencing is recommended for verification.

Coexistence of multiple genotypes *O. tsutsugamushi* was reported for 25% of scrub typhus patients in a study population in Thailand (*7*). Mixed genotype infections of a single pathogen species in the ecosystem are common; these mixed genotypes provide possibilities for recombination events and prompts the coevolution of host–parasite interactions (*8*). Disease severity and epidemiologic characteristics could be influenced by

Table. Nucleotide sequence homologies of partial 56-kDa type-specific antigen gene sequences among *Orientia tsutsugamushi* genotypes and reference strains, China, 2011

| Genotype | *O. tsutsugamushi* reference strains, % identity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | HSX | Sdu-2 | Gilliam | Karp | Kato | Kawasaki | TA686 | HSB1 |
| ZZF-KW | 100 | 68.8 | 90.3 | 72.7 | 73.8 | 96.0 | 67.3 | 74.7 |
| ZZF-HSB | 68.8 | 100 | 68.1 | 72.1 | 66.6 | 67.7 | 77.8 | 74.3 |

ecologic interactions between genetically diverse strains (*8*). In this report, we describe a single case; comparison of disease severity of scrub typhus caused by mixed and single genotype infections should be studied further.

Simultaneous infection with multiple antigenic strains of *O. tsutsugamushi* was detected in an individual mite, *Leptotrombidium arenicola* (*9*), a probable vector of scrub typhus. Infection with multiple *O. tsutsugamushi* strains may be caused by being bitten by multiple mites or by multiple genotypes coexisting within individual mites (*7*). We ascribed the co-infection to the second cause because the 2 genotypes were simultaneously detected from an eschar sample associated with the bite of 1 mite examined in this study. There may be diverse genotypic co-infection patterns of *O. tsutsugamushi*. Mechanisms of in-host interactions between genetically diverse strains of *O. tsutsugamushi* and the initiated host response require the establishment of animal models for further research.

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## *Staphylococcus delphini* and Methicillin-Resistant *S. pseudintermedius* in Horses, Canada

**To the Editor:** *Staphylococcus aureus* is a well-known pathogen of horses (*1*), but the role of other coagulase-positive staphylococcal species in these animals is unclear. *S. pseudintermedius* and *S. delphini*, members of the *S. intermedius* group (SIG), cause infections in some companion animals and equids (*2*), can be multidrug resistant, and could be a concern in horses. Members of SIG are difficult to differentiate by using biochemical methods and require molecular techniques for accurate species-level identification (*3*); therefore, misidentification of these pathogens could occur.

Methicillin-resistant or unusual staphylococci that are isolated at the Ontario Veterinary College Health Sciences Centre by the University of Guelph Animal Health Laboratory (AHL) routinely undergo further characterization. During 2011, the laboratory tested 5 isolates from different horses that were coagulase-positive staphylococci other than methicillin-resistant *S. aureus* (MRSA). Isolates were identified by using matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry, *S. pseudintermedius* or *S. delphini* PCR (*4*), and *sod*A sequence analysis (*3*). Isolates were further characterized, as indicated, by direct repeat unit typing (*5*), pulsed-field gel electrophoresis (PFGE) (*6*), *mecA* PCR (*7*), penicillin-binding protein 2a latex agglutination test, and antimicrobial drug susceptibility testing by broth microdilution and/or disk diffusion. A search of AHL's database was performed to identify other *S. pseudintermedius* and *S. delphini* isolates for all submissions of samples from equids during January 2011–August 2012.

Of the 5 isolates from the horses, 1 was identified as methicillin-resistant *S. pseudintermedius* (MRSP) and 4 as methicillin-susceptible *S. delphini* (Table). The MRSP isolate was classified by direct repeat unit typing as dt11a, a predominant MRSP clone in dogs in North America (*8*). In addition to β-lactams, the MRSP isolate was resistant to chloramphenicol, clindamycin, erythromycin, gentamicin, tetracycline, and trimethoprim/sulfamethoxazole and susceptible to nitrofurantoin, rifampin, streptomycin, and vancomycin.

The 4 *S. delphini* isolates were initially identified biochemically as *S. pseudintermedius* but subsequently classified as group A (n = 1) and group B (n = 3) *S. delphini* by molecular methods (Table). One isolate (SD-4) was resistant to only erythromycin; the remaining isolates were susceptible to all tested antimicrobial drugs. PFGE showed that 2 of the *S. delphini* isolates (SD-1 and SD-2) were possibly related, with a 4-band difference. The remaining isolates were unrelated to each other and the 2 related isolates. Two of the horses (sources of isolates SD-2 and SD-3) had been recently acquired at the same auction and were sampled on the same day; however, PFGE showed that these samples were not related and came from different groups (A, B). No common epidemiologic links were identified for any of the horses.

The AHL database search identified 8 additional horses from which *S. pseudintermedius* was biochemically identified; on the basis of drug-resistance patterns, 6 (75%) of these isolates were determined to be MRSP (Table). One additional *S. delphini* isolate was identified by using MALDI-TOF. No common epidemiologic links were identified for these infections.

MRSP is an emerging pathogen in dogs and cats (*1*) but has been rarely identified in horses (*2*). The role of these bacteria in disease in horses is unclear, but given their ability to cause opportunistic infections in other species, these pathogens should not be dismissed. *S. pseudintermedius* rarely causes disease in humans (*9*), and transmission normally occurs from infected or colonized animals. Although rarely reported, infection with MRSP might be overlooked in horses; misidentification as *S. aureus* is possible if laboratories assume that coagulase-positive staphylococci from horses are *S. aureus*, and misidentification as methicillin susceptible is possible because the use of cefoxitin susceptibility and *S. aureus* breakpoints is ineffective for determination of methicillin resistance in *S. pseudintermedius* (*10*). Additionally, *S. pseudintermedius* generates coagulase-positive results by tube testing but coagulase-negative results by slide testing, which creates the potential for misidentification as coagulase-negative staphylococci. Given the rapid expansion of *S. pseudintermedius* infections among dogs, the potential for zoonotic transmission, and the highly resistant nature of this pathogen, ongoing surveillance is indicated in the equine population.

Recently, *S. delphini* has been divided into groups A and B (*3*). The typical hosts for group A are believed to be mustelidae (i.e., mink, ferret, badger), whereas hosts for group B

Table. Results of investigation of *Staphylococcus delphini* and *S. pseudintermedius* infection in horses, Canada*

| Isolate ID or source | Species | Animal age and status | Medical history | Sample source | Date sampled | Mixed infection? | Identification method† |
|---|---|---|---|---|---|---|---|
| MRSP-1 | MRSP, dt11a | 1 y, filly | Sinusitis | Frontal sinus surgery | 2011 Aug | Yes | A |
| SD-1 | *S. delphini* (group B) | 8 y, mare | Chronic otitis externa | Ear canal swab | 2011 Feb | Yes | B |
| SD-2 | *S. delphini* (group B) | 5 y, mare | *Streptococcus equi* surveillance | Nasopharyngeal wash | 2011 Jun | No | B |
| SD-3 | *S. delphini* (group A) | 5 y, mare | *S. equi* surveillance | Nasopharyngeal wash | 2011 Jun | No | B |
| SD-4 | *S. delphini* (group B) | 4 y, mare | *S. equi* surveillance | Nasopharyngeal wash | 2011 Jul | No | B |
| AHLD | MRSP | 4 y, mare | Cough | Respiratory tract | 2011 Mar | Yes | C |
| | MRSP | 24 y, UNK | Dermatitis (pastern) | Skin (pastern) | 2011 Apr | No | C |
| | *S. pseudintermedius* | 6 y, gelding | Chronic draining abscess | Wound | 2011 May | No | C |
| | MRSP | 8 y, mare | Nasal/sinus swelling | Upper respiratory tract | 2011 Jun | Yes | C |
| | MRSP | 19 y, mare | Previous uterine infection | Uterus | 2011 Jun | Yes | C |
| | MRSP | 11 y, gelding | Draining sores on neck | Neck wound | 2011 Jul | No | C |
| | MRSP | 4 y, mare | Wound (coronary band) | Coronary band wound | 2011 Jul | Yes | C |
| | *S. pseudintermedius* | UNK, mare | Prebreeding examination | Uterus | 2011 Aug | No | C |
| | *S. delphini* | 3 mo, filly | Chronic pneumonia | Nasal passage | 2012 Jun | No | D |

*ID, identification; MRSP, methicillin-resistant *S. pseudintermedius*; AHLD, Animal Health Laboratory Database; UNK, unknown.
†A, matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF), MRSP PCR, direct repeat unit typing, *mecA* PCR, penicillin-binding protein 2a latex agglutination test, broth microdilution; B, MALDI-TOF, *S. delphini* PCR, *sodA* sequence analysis, broth microdilution; C, standard biochemical methods, disk diffusion; D, MALDI-TOF, disk diffusion.

remain unknown. *S. delphini* has rarely been identified in horses, but, as we observed, it may be misidentified by conventional methods. Although colonization or contamination appeared most likely in the instances we describe, these findings suggest that this opportunistic pathogen can be found in horses and might be pathogenic in certain situations.

Our findings highlight the importance of using additional identification methods (e.g., MALDI-TOF, *Staphylococcus* species–specific PCR) for differentiation of SIG members (notably *S. delphini* and *S. pseudintermedius*) to effectively document the emergence of these species in horses. In addition, these findings indicate the need to ensure proper differentiation of *S. aureus* from SIG in equine isolates, despite the historical predominance of *S. aureus*, because of the differences in methods for determination of methicillin resistance. Future studies are needed to determine prevalence trends and disease roles for these species in equids.

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# Kala-azar and Post–Kala-azar Dermal Leishmaniasis, Assam, India

**To the Editor:** Kala-azar (visceral leishmaniasis) is a fatal disease caused by a protozoan parasite *Leishmania donovani* and transmitted by the female sandfly, *Phlebotomus argentipes*. In the state of Assam, India, kala-azar epidemics occurred during 1875–1950 and resulted in thousands of deaths in the districts of Kamrup, Garo Hills, Goalpara, and Nagaon (*1*,*2*). The disease gradually disappeared from Assam because of the extensive use of DDT in the national malaria elimination program, and results of later entomologic studies indicated that there were no *P. argentipes* sandflies in this region after DDT use (*3*). However, sporadic kala-azar cases appeared again in Assam in 2004 (*4*), and in 2008, we reported a kala-azar outbreak in Kamrup (*5*), where kala-azar epidemics had occurred during the 1870s (*1*).

At bimonthly intervals during 2012, we conducted house-to-house surveys in 4 villages in the district of Kamrup, for a total of 845 households and 4,376 persons. Residents are socioeconomically poor and depend on agriculture and nearby brick kiln industries for their livelihood; persons involved in these industries generally keep cattle, and areas of cow manure provide breeding sites for sandflies. Persons reported with fever for >2 weeks, anemia, weight loss, and palpable spleen or liver and who were negative for malaria were tested for kala-azar by using the rK39 diagnostic kit (InBiOS, Seattle, WA, USA). We obtained bone marrow biopsy samples from selected persons who exhibited the symptoms listed above. A total of 162 persons had positive kala-azar results according to rK39

testing during 2008–2012; of these, 44 (27%) were children. Microscopic examination of bone marrow biopsy samples from 5 persons showed *L. donovani* parasites. We treated kala-azar case-patients with sodium stibogluconate (SSG). During the survey we recorded 4 suspected cases of post–kala-azar dermal leishmaniasis (PKDL).

Case-patient 1, a 16-year-old boy (panel A in online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/3/13-0260.pdf), was reported positive by rK39 for kala-azar in November 2008. After receiving 30 injections of SSG (20 mg/kg body weight), he became afebrile and his spleen decreased to a nonpalpable size. He gained weight, and hemoglobin improved to reference range. Three years after treatment, hypopigmented macules developed on his face, abdomen, and hands.

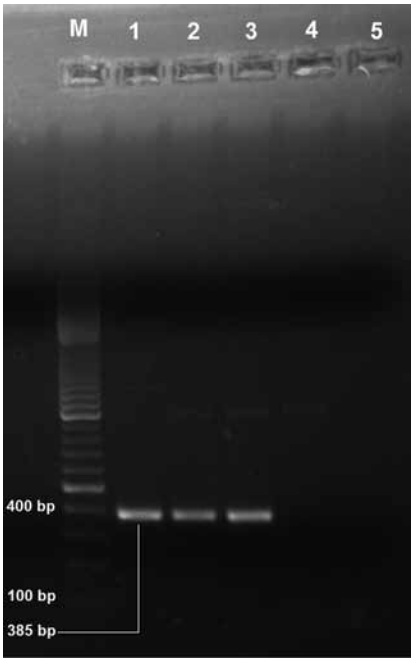


Figure. Electrophoretogram of *Leishmania donovani* kinetoplast DNA-specific PCR products (385 bp) isolated from patients with suspected post–kala-azar dermal leishmaniasis, Assam, India. Lane M, 100-bp DNA ladder; lanes 1–4, suspected post–kala-azar dermal leishmaniasis case-patients; lane 5, negative control. PCR products were visualized by staining with ethidium bromide after electrophoresis on a 1% agarose gel.

Case-patient 2 was an 18-year-old woman (online Technical Appendix Figure, panel B). Kala-azar was diagnosed in 2011, and she received 30 injections of SSG. One 1 year after completing treatment, hypopigmented macules developed on her face and hands.

Case-patient 3 was a 16-year-old girl (online Technical Appendix Figure, panel C). In 2008, after test results for kala-azar were positive, she received 30 SSG injections and clinically recovered. Macular hypopigmentation developed on her face and body 3.5 years after treatment.

Case-patient 4, a 45-year-old man (online Technical Appendix Figure, panel D), was found positive for kala-azar in 2008 and received 17 doses of SSG. He had discontinued treatment because signs and symptoms subsided considerably, and he became afebrile.

Case-patients 1–4 were clinically examined to exclude other dermal diseases caused by fungi, vitiligo, and leprosy. These persons were also tested, and found to be negative, for tuberculosis, hepatitis C virus, and hepatitis B surface antigen. We obtained punched skin biopsy samples from each case-patient; a pinch of biopsy samples were dab smeared on glass slides for examination for *L. donovani* parasites, and remaining samples were stored in RNA*later* (QIAGEN, Hilden, Germany). We microscopically examined Giemsa-stained slides and found *L. donovani* parasite in 1 sample. Using QIAamp DNA Mini Kit (QIAGEN), we isolated parasite DNA from the samples and used it for the first round of PCR with primers 5′-AAATCGGCTCCGAGGCGGGAAAC-3′ and 5′-GGTACACTCTATCAGTAGCAC-3′ as described by Salotra et al. (*6*). Primers encompassing a 385-bp fragment internal to the 592-bp of *L. donovani* minicircle kinetoplast DNA having sequence 5′-TCGGACGTGTGTGGATATGGC-3′ and 5′-CCGATAATATAGTATCTCCCG-3′ (*7*) were used for nested PCR. Three samples were positive (Figure). We treated PKDL case-patients with amphotericin B deoxycholate in accordance with World Health Organization guidelines (*8*), and these patients recovered clinically.

Resurgence of kala-azar in the Kamrup district after a 60-year absence poses new challenges to India's kala-azar elimination program. Of the 162 kala-azar cases detected, many were in children who had no history of visiting other kala-azar–endemic areas. These findings suggest local transmission of infection and are supported by the presence of the vector sandfly during the 2008 outbreak (*5*).

In India, PKDL develops in 5%–15% of treated kala-azar case-patients (*9*); in Sudan, conversion of kala-azar to PKDL is as high as 50% (*10*). PKDL cases act as reservoirs for kala-azar. Therefore, effective control depends on active surveillance for kala-azar and PKDL and treatment of kala-azar with antileishmanial drugs in accordance with Government of India guidelines (www.nvbdcp.gov.in/Doc/Guidelines-Diagnosis-Treatment-KA.pdf, www.nvbdcp.gov.in/Doc/PKDL-Guidelines-220512.pdf). Ecologic conditions of the areas where kala-azar outbreaks occurred are conducive to sandfly breeding; thus, regular spraying of DDT is needed. Preventive measures to control spread of kala-azar to other areas of Assam would be an effective step for the kala-azar control program.

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# Septic Arthritis Caused by *Streptococcus suis* Serotype 5 in Pig Farmer

**To the Editor:** *Streptococcus suis* primarily infects pigs, but >700 human infections have been reported (*1*). Cases in human occur mainly in persons who have contact with pigs; these infections are most frequently reported in Southeast Asia (*1*). In humans, *S. suis* most often causes meningitis, but endocarditis, pneumonia, toxic shock–like syndrome, and septic arthritis have also been reported (*1*–*3*).

*S. suis* is classified into serotypes on the basis of the polysaccharide capsule. Among pigs, many serotypes cause severe infections, but nearly all human cases have been attributed to serotype 2 (*1*,*3*). Other serotypes have been isolated from humans only in a few cases: meningitis caused by serotype 4 (*2*); fatal bacteremia caused by serotype 16 (*4*); sepsis caused by serotype 24 (*5*); bacteremia, meningitis, and endocarditis caused by serotype 14 (*6*–*8*); and spontaneous bacterial peritonitis caused by serotype 5 (*5*). Here, we report a case of septic arthritis caused by *S. suis* serotype 5.

The patient was a 65-year-old pig farmer who had cut his hand at work; he had not noted cases of severe illness among his pigs. He had a history of benign hyperplasia of the prostate gland, and 1 year before the current illness, he received a diagnosis of right-sided coxarthrosis, for which radiographic imaging showed grade II changes, loss of cartilage, and subchondral sclerosis. One week after the patient cut his hand, his right hip became increasingly painful, and he sought treatment at a hospital. On examination, the trochanter major region was tender (not noted at previous examinations), and passive movement of the hip was painful. Blood test results showed a slight elevation of C-reactive protein (CRP), to 31 mg/L (reference <5 mg/L). The symptoms were interpreted as trochanteritis, and treatment with nonsteroidal anti-inflammatory medication was instituted. The next day, the patient returned to the hospital with worsened pain and was admitted. He had a temperature of 37.7°C and a heart rate of 80 beats/min; blood test results showed a leukocyte count of $11.2 \times 10^9$ cells/L and CRP of 127 mg/L. Radiologic images of the hip were unremarkable, but ultrasonography-guided joint puncture showed pus and blood in the synovial fluid. Cultures were secured, and gram-positive cocci in short chains were noted in all blood culture bottles and in the synovial fluid culture. Treatment with intravenous cefotaxim was started.

Microbiological diagnosis of *S. suis* infection was made on the basis of colony morphology, a weak reaction with Lancefield anti-D antiserum, and a score of 2.31 according to matrix-assisted laser desorption/ionization–time of flight mass spectrometry (Biotyper version 3.0 software; Bruker Daltonics, Bremen, Germany). On the fourth day after admission, treatment was changed to benzylpenicillin (3 g 3×/d). The pain from the hip gradually declined, and CRP peaked at 337 mg/L on the fifth day after admission. On the seventh day after admission, treatment was changed to oral penicillin (2 g 3×/d) and was continued for 6 weeks.

At follow-up 6 months after the initial illness onset, the impairment in the patient's hip movement had worsened. Radiologic imaging showed necrosis of the femoral head, and the patient underwent total hip replacement surgery. During surgery, no signs of synovitis were noted, and 5 intraoperative cultures were negative. The procedure was completed without complications, and the patient's symptoms resolved.

The *S. suis* isolate from the patient was determined to be serotype 5 by Statens Serum Institut (SSI;

Hillerød, Denmark) by agglutination with latex beads and type-specific serum and by microscopic determination of capsule swelling with type-specific serum (SSI Diagnostika, Hillerød, Denmark), according to the manufacturer's instructions. These methods gave concordant results. Etests (bioMérieux, Solna, Sweden) demonstrated that the isolate was sensitive to all antimicrobial drugs tested; MIC was 0.125 mg/L for cefotaxim and 0.016 mg/L for benzylpenicillin. The isolate was tested for known virulence-associated genes *sly*, *mrp*, and *epf* with PCR, as described (*9*). PCR fragments of predicted sizes were obtained with primer hybridizing to *sly* and *mrp* but not with primers hybridizing to *epf*. A serotype 2 isolate (kindly provided by Susanne Sauer at SSI) was used as a positive control for the *epf* primers.

*S. suis* is an emerging human pathogen, but reports of human infections caused by serotypes other than serotype 2 remain scarce. This case demonstrates that *S. suis* of serotype 5, which is a serotype routinely isolated from deceased pigs (*10*), can cause invasive infections in humans. The course of the described infection was relatively favorable, and the patient did not show signs of a systemic inflammatory response syndrome or of meningitis. Preexisting osteoarthritis of the right hip might have had diminished local defenses, thereby enabling colonization of the hip area by bacteria that had entered the bloodstream through the wound on the patient's hand. The isolate we recovered possessed *sly* and *mrp* genes, which encode the virulence-associated suilysin and muraminidase-released proteins, but clearly other factors are also of importance for determining the virulence of individual *S. suis* isolates.

The patient gave his informed consent to the writing of this article.

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## *Bartonella henselae* and *B. koehlerae* DNA in Birds

**To the Editor**: Bartonellosis, a globally emerging vector-borne zoonotic bacterial disease, is caused by hemotropic, gram-negative, aerobic, facultative intracellular *Bartonella* spp. (*1*). Of the 30 *Bartonella* species/subspecies, 17 have been associated with human infections (*2*,*3*). Each species has a reservoir host(s), within which the bacteria can cause intraerythrocytic bacteremia with few or no clinical signs of illness (*1*,*3*); the bacteria are transmitted by hematophagous arthropod vectors (*1*). Various *Bartonella* spp. have been identified in domestic and wild animals, including canids, deer, cattle, rodents, and marine mammals (*1*,*4*). *Bartonella* DNA from the blood of loggerhead sea turtles (*Caretta caretta*) has been PCR amplified and sequenced (*5*); the fact that *Bartonella* DNA was found suggests the possibility that persistent blood-borne infection can occur in nonmammals and that the host range for *Bartonella* spp. may be larger than anticipated.

Growing evidence suggests that wild birds play key roles in the maintenance and movement of zoonotic pathogens such as tick-borne encephalitis virus and *Borrelia* and *Rickettsia* spp. (*6*–*9*). *Bartonella grahamii* DNA was amplified from a bird tick in Korea (*10*). The substantial mobility, broad distribution, and migrations of birds make them ideal reservoir hosts for dispersal of infectious agents.

To investigate whether birds might be a reservoir for *Bartonella* spp., we screened 86 birds for the presence of *Bartonella* spp. DNA.

The primary study site was a residential backyard in Morehead City, North Carolina, USA (34°43.722′N, 76°43.915′W). Of the 86 birds screened, 78 (16 species) were captured by mist net during March 2010–June 2012 and 8 (3 species) were injured birds that were to be euthanized (Table). Each bird was examined for external abnormalities and ectoparasites, weighed, measured, and tagged with a US Geological Survey–numbered band. A blood sample (0.10–0.25 mL) was collected from each bird by using a 1-mL insulin syringe with a 28-gauge × 1.27-cm needle. Blood remaining after preparation of blood smears was added to an EDTA tube and frozen (–80°C) until processed. Blood smears were examined for hemoparasites. Research was conducted under required state and federal bird banding permits and with the approval of the North Carolina State University Institutional Animal Care and Use Committee.

Before DNA was extracted from the samples, 10 μL of blood was diluted in 190 μL of phosphate-buffered saline. DNA was automatically extracted by using a BioRobot Symphony Workstation and MagAttract DNA Blood M96 Kit (QIAGEN, Valencia, CA, USA). *Bartonella* DNA was amplified by using conventional *Bartonella* genus PCR primers targeting the 16S–23S intergenic spacer region: oligonucleotides, 425s (5′-CCG GGG AAG GTT TTC CGG TTT ATCC-3′) and 1,000as (5′-CTG AGC TAC GGC CCC TAA ATC AGG-3′). Amplification was performed in a 25-mL reaction, as described (*3*). All PCR reactions were analyzed by 2% agarose gel electrophoresis. Amplicons were sequenced to identify the *Bartonella* sp. and intergenic spacer region genotype. To compare sequences with those in GenBank, we identified bacterial species and genotypes by using Blast version 2.0 (http://blast.ncbi.nlm.nih.gov/Blast. cgi). DNA extraction and PCR-negative controls remained negative throughout the study.

Results are summarized in the Table. None of the screened birds were anemic, but 5 were PCR positive for *Bartonella* spp. (3 for *B. henselae* and 2 for *B. koehlerae*). *B. henselae* was amplified from 2 Northern Mockingbirds (*Mimus polyglottos*) and 1 Red-winged Blackbird (*Agelaius phoeniceus*) (GenBank accession no. KC814161). The DNA sequences were identical to each other and had 99.6% (456/457 bp) sequence similarity with *B. henselae* San Antonio 2 intergenic spacer region genotype (GenBank accession no. AF369529). *B. koehlerae* was amplified from a Red-bellied Woodpecker (*Melanerpes carolinus*) and a Common Loon (*Gavia immer*) (GenBank accession no. KC814162). The DNA sequences were identical to each other (404/404 bp) and to GenBank sequence AF312490. Lice (Mallophaga order) were found on 5 Boat-tailed Grackles (*Quiscalus major*), but no ectoparasites were observed on *Bartonella* spp.–positive birds. Hemoparasites (*Haemoproteus* and *Plasmodium* spp.) were detected in 7 of 86 birds, indicating exposure to hematophagous ectoparasites, but hemoparasites were not detected in the *Bartonella* spp.–positive birds. No bacteria were visualized in *Bartonella* PCR–positive blood smears.

*Bartonella* spp. are increasingly associated with animal and human illnesses; thus, the identification of reservoirs and increased understanding of *Bartonella* spp. disease ecology are of public health importance. Our finding of 2 pathogenic species not previously reported in birds has expanded the potential sources for zoonotic infection.

There is growing evidence that migratory birds serve as reservoirs

Table. *Bartonella* species detected in birds*

| Bird common name | Bird species | No. birds positive/no. total | *Bartonella* sp. |
|---|---|---|---|
| House sparrow | *Passer domesticus* | 0/28 | |
| Boat-tailed grackle | *Quiscalus major* | 0/15 | |
| Mourning dove | *Zenaida macroura* | 0/12 | |
| Herring gull† | *Larus argentatus* | 0/6 | |
| House finch | *Carpodacus mexicanus* | 0/5 | |
| Blue jay | *Cyanocitta cristata* | 0/3 | |
| Song sparrow | *Melospiza melodia* | 0/2 | |
| Northern cardinal | *Cardinalis cardinalis* | 0/2 | |
| Northern mockingbird | *Mimus polyglottos* | 2/2 | *B. henselae* SA2 |
| European starling | *Sturnus vulgaris* | 0/2 | |
| Red-winged blackbird | *Agelaius phoeniceus* | 1/1 | *B. henselae* SA2 |
| Brown thrasher | *Toxostoma rufum* | 0/1 | |
| Tufted titmouse | *Baeolophus bicolor* | 0/1 | |
| Red-bellied woodpecker | *Melanerpes carolinus* | 1/1 | *B. koehlerae* |
| Common grackle | *Quiscalus quiscula* | 0/1 | |
| Common loon† | *Gavia immer* | 1/1 | *B. koehlerae* |
| Red-headed woodpecker | *Melanerpes erythrocephalus* | 0/1 | |
| Brown pelican† | *Pelicanus occidentalis* | 0/1 | |
| Collared dove | *Streptopelia decaocto* | 0/1 | |

*SA2, San Antonio 2 intergenic spacer region genotype.
†Euthanized.

and/or mechanical vectors for pathogens such as tick-borne encephalitis virus and *Rickettsia* spp. (*6*–*8*). Birds have been implicated as reservoirs for several *Borrelia* spp. (*9*,*10*) and for possible dispersion of other tick-borne pathogens (e.g., *Anaplasma* and *Bartonella* spp.) (*6*,*10*). Tick transmission of *Bartonella* spp. to birds should be investigated, and additional studies that investigate the reservoir host range of *Bartonella* spp. and the transmission of these bacteria to non–host species will improve epidemiologic understanding of bartonellosis and will identify additional risk factors for *Bartonella* spp. transmission to new hosts, including humans.

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# Tick-borne Pathogens in Northwestern California, USA

**To the Editor:** In northwestern California, USA, the western blacklegged tick, *Ixodes pacificus*, is a known vector of *Borrelia burgdorferi*, the spirochete that causes Lyme disease. *B. miyamotoi,* which is more closely related to spirochetes that cause relapsing fever, has also been detected in 2 locations in California (*1*,*2*) and has recently been implicated as a human pathogen in the northeastern United States (*3*,*4*). Other studies may have unintentionally included *B. miyamotoi* infections among measures of *B. burgdorferi* if the diagnostics were for spirochetes (e.g., direct fluorescent antibody tests or dark-field microscopy) or genetically targeted for *Borrelia* spp. (*5*).

To investigate *Borrelia* spp. ecology in California, we collected adult *I. pacificus* ticks by dragging a 1-$m^2$ white flannel blanket along vegetation and/or leaf litter in 12 recreational areas in the San Francisco Bay area during January–May 2012 (Table). Habitat varied from chaparral and grassland to coastal live oak woodland. Ticks were pooled for examination by quantitative PCR (qPCR) for the presence of *Borrelia* spp. We interpreted the prevalence of *Borrelia* spp. from positive pools as the minimum infection prevalence (i.e., assuming 1 positive tick/positive pool). DNA was extracted from ticks by using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocols and then stored at –20°C until use. DNA was analyzed by qPCR, with use of primer and fluorescent hybridization probes previously developed to differentiate *Borrelia* spp. spirochetes (*5*). To identify the *Borrelia* spp. genotype, we attempted to sequence the 16S–23S (*rrs-rrlA*) intergenic spacer of each sample positive by qPCR (*8*). The nested PCR product was further purified by using the QIAquick Kit (QIAGEN) and then sequenced (Environmental Genetics and Genomics Laboratory, Northern Arizona University, Flagstaff, AZ, USA; www.enggen.nau.edu/dna.html) by using capillary Sanger sequencing on an ABI 3730 sequencer (Life Technologies, Grand Island, NY, USA). BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare each sequence to other *Borrelia* spp. sequences available from GenBank.

From a total of 1,180 adult ticks, we found 43 samples positive for *Borrelia* spp., resulting in a minimum infection prevalence of 3.6% (Table). We obtained intergenic spacer sequence data for 27 of the positive samples; 6 samples were *B. burgdorferi* sensu stricto, 7 were *B. burgdorferi* sensu lato (both on the basis of alignments of 816 bp), and 14 were *B. miyamotoi* (on the basis of alignments of 503 bp). The *B. miyamotoi* sequences for our samples from California and those for isolates from the eastern United States (*9*) and Japan (*8*) formed a monophyletic clade that was oriented as a sister clade to the 3 *Borrelia* spp. that cause tick-borne relapsing fever in the United States (*B. hermsii, B. turicatae*, and *B. parkeri*).

We found borreliae-infected adult *I. pacificus* ticks at all 12 sites from which tick sample sizes exceeded 30. When the presence of *B. burgdorferi*

Table. *Borrelia* spp. infection prevalence among adult *Ixodes pacificus* ticks in northwestern California, USA, January–May 2012*

| | No. *Borrelia* spp. ticks infected/total (%) | | | | |
|---|---|---|---|---|---|
| Location, County (reference) | *B. burgdorferi* sensu stricto | *B. burgdorferi* sensu lato | *B. miyamotoi* | Unsequenced species | All species |
| Jasper Ridge Biologic Preserve, San Mateo | | | | 1/32 (3.1) | 1/32 (3.1) |
| Pulgas Ridge OSP, San Mateo | | | | 2/118 (1.7) | 2/118 (1.7) |
| Thornewood OSP, San Mateo† | 1/156 (0.6) | 2/156 (1.3) | 2/156 (1.3) | 4/156 (2.6) | 9/156 (5.8) |
| Thornewood OSP, San Mateo‡ | | | | | 0/9 (0) |
| Windy Hill OSP, San Mateo† | 2/120 (1.7) | | | 1/120 (0.8) | 3/120 (2.5) |
| Windy Hill OSP, San Mateo§ | 2/122 (1.6) | 3/122 (2.5) | 1/122 (0.8) | 2/122 (1.6) | 8/122 (6.6) |
| Wunderlich County Park, San Mateo | | | | | 0/15 (0) |
| Foothills Park, Santa Clara | | | | | 0/13 (0) |
| Henry W. Coe State Park, Santa Clara | | | 3/132 (2.3) | | 3/132 (2.3) |
| Monte Bello OSP, Santa Clara | 1/46 (2.2) | | | 1/46 (2.2) | 2/46 (4.3) |
| Sanborn County Park, Santa Clara | | | 4/53 (7.5) | | 4/53 (7.5) |
| Sierra Azul OSP, Santa Clara | | | 2/112 (1.8) | | 2/112 (1.8) |
| Los Trancos OSP, San Mateo and Santa Clara | | | 1/58 (1.7) | 1/58 (1.7) | 2/58 (3.4) |
| Castle Rock State Park, Santa Cruz | | 1/51 (2.0) | | 2/51 (3.9) | 3/51 (5.8) |
| Castle Rock State Park, Santa Cruz (*6*) | | | | | 13/264 (4.9) |
| Tilden Regional Park, Contra Costa (*2*) | 1/814 (0.1) | | 4/814 (0.5) | | 5/814 (0.6) |
| China Camp State Park, Marin | | 1/143 (0.7) | 1/143 (0.7) | 2/143 (1.4) | 4/143 (2.8) |
| Hopland Research and Extension Center, Mendocino (*1,7*) | 4/282 (1.4) | | 2/282 (0.7) | | |
| Total (this study) | 6/1,108 (0.5) | 7/1,108 (0.6) | 14/1,108 (1.3) | 16/1,108 (1.4) | 43/1,108 (3.6) |

*Data are from this study and from previously published research (indicated by reference no.). OSP, open space preserve.
†Woodland.
‡Redwood.
§Chaparral/grassland.

sensu stricto or *B. burgdorferi* sensu lato was detected (4/12 sites each), prevalence was 0.6%–2.2% and 0.7%–2.5%, respectively. *B. miyamotoi* was detected at 7/12 sites, and prevalence ranged from 0.7% to 7.5%. A previous survey of *B. burgdorferi* in nearby Santa Cruz County recreational areas reported an infection prevalence of ≈6% among adult *I. pacificus* ticks (*6*); the study did not, however, differentiate between *Borrelia* spp. and therefore may have included *B. miyamotoi* among its prevalence measures (*5*). In our study, *B. burgdorferi* was found more frequently in woodland habitats, but it was also detected in a grassland–chaparral habitat several hundred meters from the nearest woodland. We did not detect *B. bissettii*, a species recently implicated as a human pathogen in Mendocino County, California (*10*). The high level of habitat variation in northwestern California presents a varied risk for *Borrelia*-associated tick-borne disease in humans because of diverse variations in vertebrate reservoir ecology, tick abundance, and human exposure to ticks. This variation emphasizes the need to understand the local epidemiology and ecology of a disease.

In adult *I. pacificus* ticks in the San Francisco Bay area, *B. miyamotoi* is as abundant as its congener *B. burgdorferi.* Human disease caused by *B. miyamotoi* infection has not been reported in California, and transmission efficiency of *B. miyamotoi* by *I. pacificus* ticks is unknown. However, it is possible that *B. miyamotoi* infections in ticks and humans have not been accurately diagnosed. We advocate for increased scrutiny of the eco-epidemiology of *B. miyamotoi* in human, tick, and possible vertebrate host populations in northwestern California.

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## Buruli Ulcer in Liberia, 2012

**To the Editor:** Buruli ulcer, a necrotizing skin disease caused by *Mycobacterium ulcerans*, is highly endemic to West Africa (*1*,*2*) and is characterized by large ulcerations on the lower limbs (60% of cases) as well as on the upper limbs (30%) and other parts of the body (10%). Although the mode of transmission is unknown, most cases of Buruli ulcer occur around swampy and riverine areas; children <15 years of age are most often affected (*2*,*3*). The recommended treatment consists of a combination of daily oral rifampin and intramuscular streptomycin for 8 weeks, supplemented by wound care when appropriate (*4*). Large ulcers may require debridement and grafting to facilitate wound closure, and physiotherapy is often indicated to prevent functional limitation, particularly for lesions located over joints.

In Liberia, 2 Buruli ulcer patients were reported in 1981 in the Foya region, along the Manor River basin; 4 more patients were observed in the same area in 1984 (*5*). Since then, some patients from Liberia have received treatment for Buruli ulcer in Côte d'Ivoire (*6*), and suspicious cases have been detected in some parts of Liberia since the end of a civil war in 2004.

Recently, Buruli ulcer cases have been suspected in 3 counties of Liberia, Bong, Lofa, and Nimba; these regions share borders with the Buruli ulcer–endemic regions of Côte d'Ivoire and Guinea. During 2012, the government of Liberia, with assistance from the Medical Assistance Program International and with technical support from the World Health Organization (WHO), conducted a rapid status assessment in these 3 counties. In January 2012, a core team of national and county health personnel was trained in the recognition and assessment of Buruli ulcer. Assessment was conducted during February 18–27, 2012, by a team made up of those who had received the preassessment training and WHO Buruli ulcer consultants.

During the preassessment training, notice was given to all health facilities to record all lesions with features suggestive of Buruli ulcer. The persons identified during this period then came to the nearest health facility to be examined by the assessment team or were traced to their homes. A detailed history was collected and physical examination conducted, and swab specimens and fine-needle aspirates (2 for each lesion) were obtained for confirmation of diagnosis by classical PCR (*7*).

On the basis of the WHO case definition for Buruli ulcer (*1*), 60 of 181 persons screened were suspected to have Buruli ulcer. All cases were documented by photography and registration on a modified version of the WHO Buruli ulcer assessment form (*1*). For 21 (35%) of the 60 patients, *IS2404* PCR testing at Komfo Anokye Teaching Hospital in Ghana confirmed the clinical diagnosis; these patients received the recommended treatment for Buruli ulcer. Those with negative test results received wound care and supportive management.

A total of 21 confirmed cases occurred: 9 in Nimba County, and 6 each in Bong and Lofa Counties (Figure). Nine (35%) of the 21 patients were children ≤15 years of age; 11 patients were male and 10 female. Most (17 of 21) lesions were on the lower limbs; 3 were on the upper limbs and 1 on the thorax. Fifteen patients had ulcers, 2 edema, and 3 osteomyelitis. No lesions were classified as category I, but 11 (52.4%) were category III.

Our findings suggest that Buruli ulcer in Liberia may be more prevalent than previously thought. Although only 3 of 15 counties were assessed, results show that Buruli ulcer has not disappeared from Liberia and that the absence of regular reporting should be investigated. A long civil war and lack of familiarity with the disease by health care workers may have contributed to poor reporting.

It has been almost 3 decades since the last published report of Buruli ulcer cases in Liberia (*5*), but other studies have found the disease in countries many years after it was last reported. In Cameroon, a case search in 2001 in 2 districts where cases had last been reported 24 years earlier found 436 active and inactive cases (*8*). In southwestern Nigeria, a case search in 2006 found 14 active and inactive cases 30 years after the most recent publication (*9*). More recently, in 2012, a similar situation was reported in Gabon (*10*).

Several measures might improve Buruli ulcer control and surveillance in Liberia. First, treatment and control activities should be included in the Neglected Tropical Diseases Control Program at all levels to enhance

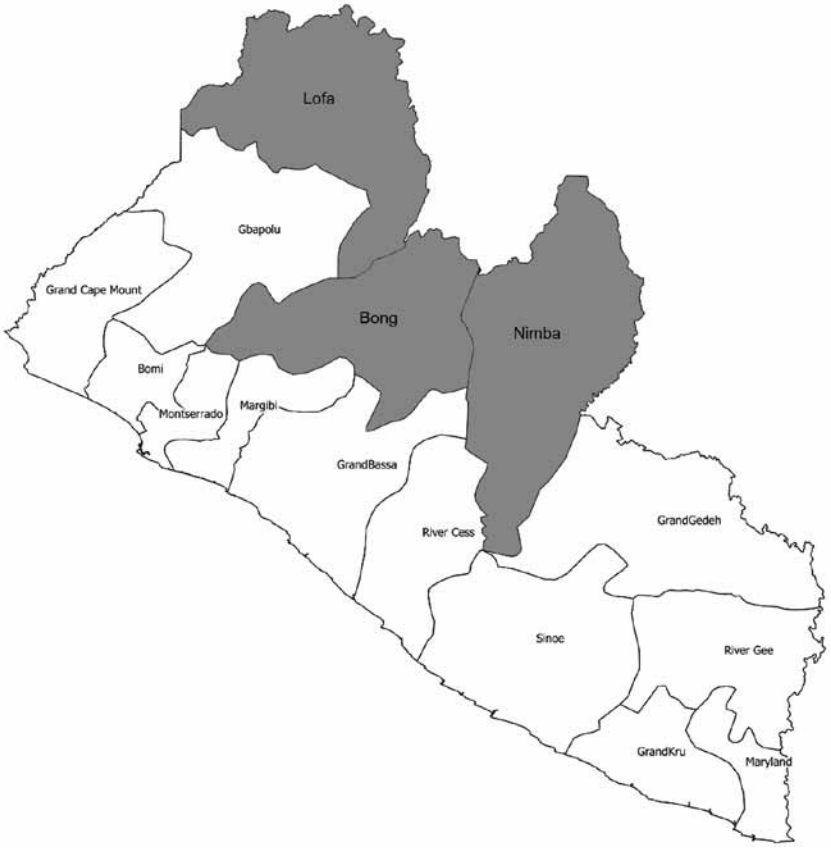


Figure. Counties in which cases of Buruli ulcer were found during 2012 (gray shading), Liberia.

surveillance. Second, health workers at all levels should be trained to recognize the disease. Third, a detailed assessment of the extent of Buruli ulcer in the 3 counties visited as well as in other counties should be prepared. Fourth, partner/donor support for Buruli ulcer activities should be enhanced. Fifth, capacity of the National Reference Laboratory to be able to perform PCR for confirmation of Buruli ulcer cases should be expanded. Last, Buruli ulcer should be incorporated into the national surveillance system to enable better data collection.

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# *Candidatus* Neoehrlichia mikurensis and *Anaplasma phagocytophilum* in Urban Hedgehogs

**To the Editor:** *Candidatus* Neoehrlichia mikurensis is a member of the order Rickettsiales, family *Anaplasmataceae* (*1*). Manifestations of infection with these bacteria are atypical and severe and include cough, nausea, vomiting, anemia, headache, pulmonary infiltration, malaise, myalgia, arthralgia, fatigue, recurrent fever for ≤8 months, and/or death (*2–5*). *Candidatus* N. mikurensis has been detected in *Ixodes ovatus*, *I. persulcatus*, and *Haemaphysalis concinna* ticks in Asia (*1,5*).

*Candidatus* N. mikurensis has been identified as one of the most prevalent pathogenic agents in *I. ricinus* ticks throughout Europe (*2,3,6*). Rodents of diverse species and geographic origins have been shown to carry these bacteria, but transmission experiments have not been conducted to unambiguously identify natural vertebrate reservoirs (*1–3,5–7*). This emerging tickborne pathogen has been detected mainly in immunocompromised patients in Sweden (n = 1), Switzerland (n = 3), Germany (n = 2), and the Czech Republic (n = 2) and in immunocompetent patients in China (n = 7) (*2–5*).

*Anaplasma phagocytophilum* is an obligate, intracellular, tickborne bacterium of the family *Anaplasmataceae* and causes granulocytic anaplasmosis in humans and domestic animals. In Europe, *I. ricinus* ticks are its major vector, and red deer, roe deer, rodents, and European hedgehogs (*Erinaceus europaeus*) are suspected reservoir hosts (*8*).

Northern white-breasted hedgehogs (*Erinaceus roumanicus*) are urban-dwelling mammals (order Eulipotyphla, family Erinaceidae) that serve as major maintenance hosts for the 3 stages of

*I. ricinus* ticks (*9*). However, *E. roumanicus* hedgehogs have not been studied for their ability to carry *A. phagocytophilum*. In addition, no suspected reservoirs other than rodents have been investigated for *Candidatus* N. mikurensis. The purpose of this study was to determine whether this hedgehog is a potential reservoir of these 2 bacteria.

We conducted an ecoepidemiologic study during 2009–2011 to obtain information about ticks and tickborne pathogens of urban hedgehogs in a park on Margaret Island in central Budapest, Hungary (*9*). Ear tissue samples were obtained from hedgehogs anesthetized with intramuscular ketamine (5 mg/kg) and dexmedetomidine (50 μg/kg).

DNA was extracted from samples by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) or the Miniprep Express Matrix protocol (MP Biomedicals, Santa Ana, CA, USA). We used quantitative real-time PCRs that partially amplify the heat shock protein gene (*groEL*) of *Candidatus* N. mikurensis and the major surface protein 2 gene (*msp2*) of *A. phagocytophilum* (*3*). PCR was performed in a 20-μL volume containing iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, CA, USA) in a LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche, Basel, Switzerland). Final PCR concentrations were 1× iQ Powermix, 250 nmol/L of primers ApMSP2F and ApMSP2R, 125 nmol/L of probe ApMSP2P-FAM, 250 nmol/L of primers NMikGroEL-F2a and NMikGroEL-R2b, 250 nmol/L of probe NMikGroEL-P2a-RED, and 3 μL of template DNA.

To confirm quantitative PCR results, we performed conventional PCRs in a Px2 Thermal Cycler (Thermo Electron Corporation, Waltham, MA, USA) on selected PCR-positive samples for both pathogens (*3*). Sequences obtained were submitted to GenBank under accession nos. KF803997 (*groEL* gene of *Candidatus* N. mikurensis) and KF803998 (*groEL* gene of *A. phagocytophilum*).

*Candidatus* N. mikurensis was detected in 2 (2.3%) of 88 hedgehog tissue samples. Formerly, rodents were the only wild mammals found to act as potential reservoirs for this pathogen. Results of studies that attempted to detect these bacteria in common shrews (*Sorex araneus*), greater white-toothed shrews (*Crocidura russula*) (*2,3*), or common moles (*Talpa europaea*) (*2*) were negative. However, our results indicate that northern white-breasted hedgehogs might be a non-rodent reservoir for *Candidatus* N. mikurensis.

The low pathogen prevalence observed in this urban hedgehog population compared with that in rodents in other locations (*2,3*) might be caused by use of skin samples. Skin samples from rodents showed only 1.1% positivity in a study in Germany; however, average prevalence of *Candidatus* N. mikurensis in transudate, spleen, kidney, and liver samples from the same animals was 37.8%–51.1% (*2*). Although we did not test other organs, we hypothesize that prevalence of *Candidatus* N. mikurensis infection in urban hedgehogs is probably >2.3%.

We detected *A. phagocytophilum* in 67 (76.1%) of 88 urban hedgehogs. This prevalence was similar to that found among European hedgehogs in Germany (*8*). *I. ricinus* ticks are more common than *I. hexagonus* ticks in this urban hedgehog population (*9*). Thus, *I. ricinus* ticks can acquire these bacteria when feeding on hedgehogs and the risk for human infection with *A. phagocytophilum* in this park in Budapest is relatively high.

Neoehrlichiosis and granulocytic anaplasmosis have not been diagnosed in humans in Hungary. This finding is probably caused by diagnostic difficulties rather than absence of these pathogens in the environment. Infection with *Candidatus* N. mikurensis and *A. phagocytophilum* cause predominantly noncharacteristic symptoms. Laboratory cultivation and serologic detection of *Candidatus* N. mikurensis has not been successful, and this pathogen has not been identified in blood smears. Thus, accurate diagnosis of suspected cases requires suitable molecular methods.

Parks can be considered points of contact for reservoir animals, pathogens, ticks, and humans. Our results indicate that *E. roumanicus* hedgehogs play a role in urban ecoepidemiology of ≥2 emerging human pathogens. To better understand the urban cycle of these pathogens, potential reservoir hosts, ticks collected from these hosts, and vegetation in parks should be investigated.

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# *Rickettsia* and Vector Biodiversity of Spotted Fever Focus, Atlantic Rain Forest Biome, Brazil

**To the Editor:** *Rickettsia rickettsii*, *R. felis*, and *R. parkeri*, strain Atlantic rainforest, have been characterized after being found in areas to which Brazilian spotted fever (BSF) is endemic (*1*,*2*), which indicates the complexity of their epidemic and enzootic cycles. The Atlantic rain forest is one of the largest and richest biomes of Brazil, and antropic action has intensely influenced its transformation. Most BSF cases and all BSF-related deaths are recorded in this biome area.

Many BSF cases were recorded in Paraíba do Sul river basin, one of the most urbanized and industrialized areas of Brazil. To better understand arthropod and *Rickettsia* diversity in this area,, we analyzed 2,076 arthropods from Rio de Janeiro state, Atlantic rain forest biome.

During October 2008–November 2009, we collected ticks and fleas from hosts and environments in 7 cities where high numbers of BSF cases were recorded (Rio de Janeiro State Health Secretary, unpub. data) and where fisiogeographic characteristics differed. After morphologic classification (*3*), the arthropods were individually separated or grouped by sex, developmental stage, and host for total DNA extraction (*4*).

We used 2 *Rickettsia*-specific primer sets (CS2–78/CS2–323 and CS4–239/CS4–1069) to amplify 401 bp and 834 bp, respectively, of the citrate synthase gene (*gltA*) (*5*,*6*). Presumptive *Rickettsia*-positive samples were tested for spotted fever group (SFG)–specific primer set *Rr*190.70p/*Rr*190.602n for 532 bp from the *ompA* gene (*7*). *R. rickettsii* DNA and bi-distilled water were used as positive and negative controls, respectively. PCR products were purified (NucleoSpin Extract II kit; Macherey-Nagel, Düren, Germany), cloned (pTZ57R/T; Fermentas-Thermo Fisher Scientific, Waltham, MA, USA), and sequenced by using specific vector primer sets (BigDye Reaction kit, Applied Biosystems, Foster City, CA, USA). Sequences were edited by using SeqMan program (Lasergene 10.1; DNASTAR Inc., Madison, WI, USA), and similarities were obtained by BLAST analysis (http://blast.ncbi.nlm.nih.gov). The phylogenies were assessed by applying neighbor-joining and maximum-parsimony methods, with the Kimura 2-parameter correction model. We used ClustalW 2.1 (www.clustal.org) to align sequences and produced phylogenetic trees by using 1,000 replicates bootstrap in MEGA 5.0 software (www.megasoftware.net).

We collected and analyzed ticks of the following species: *Amblyomma cajennense* (1,723 ticks), *Rhipicephalus sanguineus* (109), *Anocentor nitens* (63), *Boophilus microplus* (33), *Amblyomma aureolatum* (2), and *Amblyomma dubitatum* (2). We collected and analyzed *Ctenocephalides felis* (143 fleas) and *C. canis* (1) fleas.

PCR analysis showed Rickettsia DNA in 11 individual or pooled samples. This finding indicated minimal infection rates of 0.2% (4/1,723) for A. cajennense ticks, 50% (2/4) for A. dubitatum ticks, 3.0% (1/33) for B. microplus ticks, 100% (1/1) for C. canis fleas, and 2.8% (4/143) for C. felis fleas. Expected amplicon size, determined by using the gltA 401-bp primer set, was observed for all positive samples. Two were also positive by PCR

for gltA 834 bp and 4 for ompA primer set (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/3/13-1013-Techapp1.pdf). The sequences were deposited in GenBank; BLASTn analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi) indicates that these sequences belong to AG (ancestral) or SFG rickettsiae (Figure).

In phylogenetic inferences, 8 samples were grouped with SFG *R. rickettsii*, supported by bootstrap value >62%. In addition, 3 samples were closely related to SFG *R. felis,* strongly supported by bootstrap values >99%; *Rickettsia* sp. LIC2937Ac was closely related to AG *R. bellii* under a bootstrap support >99% (Figure).

Epidemic manifestations of rickettsial diseases vary by ecotope characteristics, human activity, and vector bioecology in natural foci. BSF is a clinically distinct rickettsial infection in foci to which it is endemic. BSF-related illness and death vary by the *Rickettsia* species that can coexist in a given area and

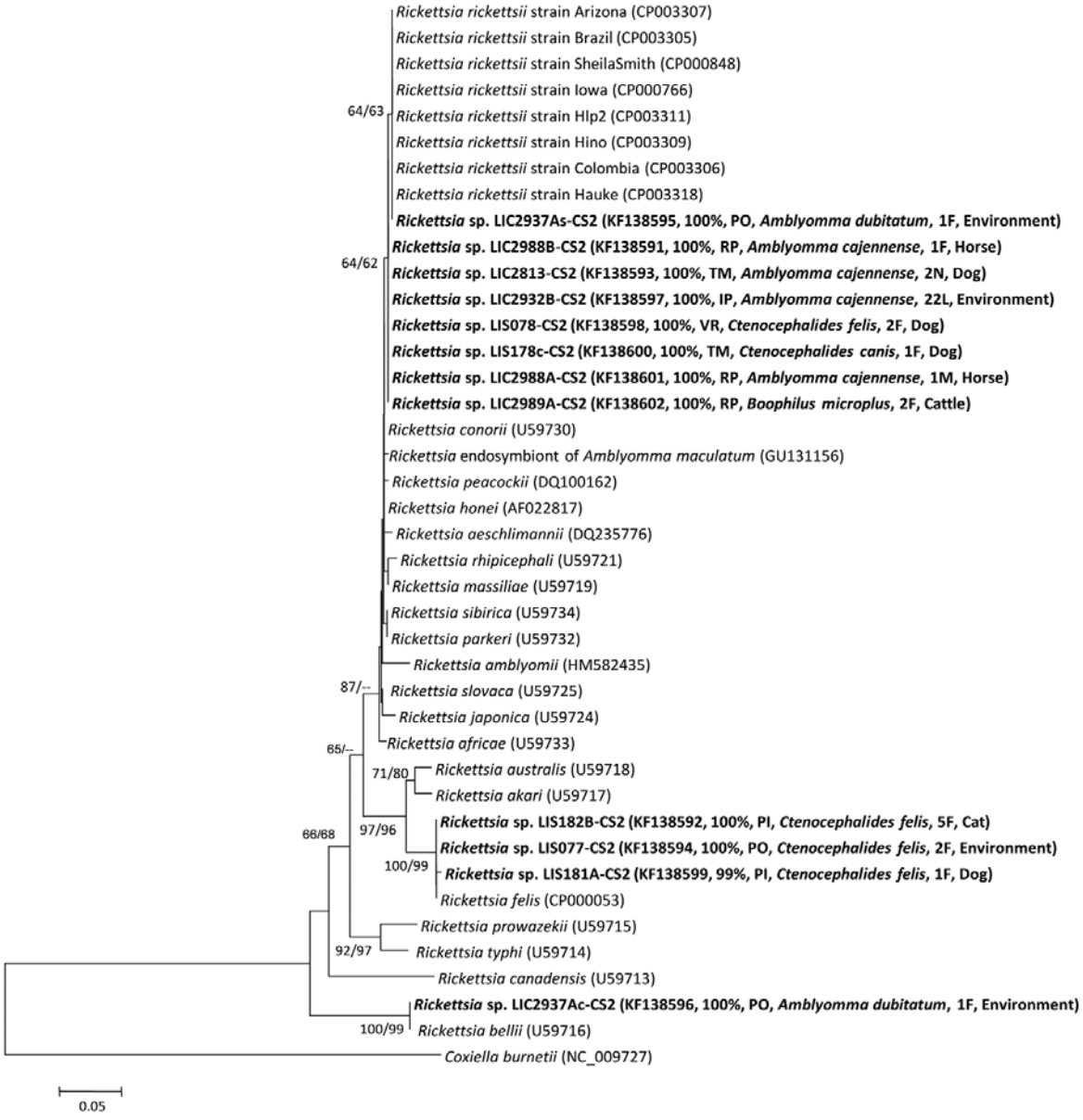


Figure. Phylogenetic inferences by neighbor-joining method from 1,000 replicated trees based on partial sequence of the *Rikettsia gltA* gene (CS2 401 bp). Evolutionary distances were estimated by the Kimura 2-parameter model. Bootstrap values >60% are shown (neighbor-joining/maximum-parsimony). Sequences obtained are in boldface, and GenBank accession numbers are in parentheses, followed by the similarity percentage (BLAST, http://blast.ncbi.nlm.nih.gov), the locality acronym (PO, Porciúncula; RP, São José do Vale do Rio Preto; TM, Trajano de Moraes; IP, Itaperuna; VR, Volta Redonda; PI, Piraí), the arthropod vector species, the composition of the sample (L, larvae; N, nymph; F, female; M, male), and the host. Scale bar indicates nucleotide substitutions per site.

that can share or not share epidemiologic elements.

Molecular identification of *R. rickettsii* in *A. cajennense* ticks was recorded only in the Paraíba do Sul River basin of southeastern Brazil (*8*), as confirmed in our study. This eco-epidemiologic aspect, its great anthropophily, and its presence in all municipalities surveyed, with absolute frequency greater than other species, demonstrates the possible effect of this tick on epidemic cycle development for the analyzed region, which does not seem to occur in other regions.

*R. rickettsii* infection of *A. dubitatum* ticks in the 1 focus analyzed might indicate its relevance in specific epidemiologic scenarios. We detected highly similar sequences of different species of *Rickettsia* (LIC2937A) in the same *A. dubitatum* tick specimen (Figure). Other studies have recorded multiple *Rickettsia* infections in 1 tick specimen (*9*,*10*).

Our finding of *C. felis* fleas in 6 of the 7 outbreaks investigated highlights the possible role of this flea in maintaining *Rickettsia* in Rio de Janeiro state. *C. felis* and *C. canis* fleas infected with *R. rickettsii* seem to confirm this potential. Nevertheless, the real epidemiologic value of this report in the BSF cycle deserves to be further investigated.

Our results indicate that dogs and horses are the primary vertebrates in the *Rickettsia* enzootic cycle in the investigated focus, and, considering their common presence in human environments, they must be important in maintaining possible rickettsial vectors to humans. These results contribute to the mapping of BSF-endemic areas and to the understanding of the circulation and epidemiology of *Rickettsia* sp. in an area with one of the highest fatal concentrations of BSF.

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## Atypical *Streptococcus suis* in Man, Argentina, 2013

**To the Editor:** *Streptococcus suis* is a major swine pathogen and an emerging zoonotic agent that causes mainly meningitis and septic shock (*1*,*2*). Among the 35 described

serotypes classified by differences in capsular antigens, serotype 2 is the most frequently isolated from humans worldwide, and serotype 14 cases are also increasing in some countries (*1*). In Southeast Asia, this pathogen affects not only workers in close contact with pig/pork by-products but also the general population, probably because of the widespread presence of backyard types of pig production, open meat markets, and some special dishes prepared with raw meat or blood (*3*). We report a case of peritonitis caused by an atypical *S. suis* serotype 21 strain in a patient in Argentina.

A 62-year-old man from Santa Fe Province in Argentina, who had a history of tobacco and alcohol abuse, was hospitalized in 2013 as an emergency patient with symptoms of acute abdominal distress. Ten days before admission, abdominal distention, accompanied by intense upper abdominal pain, developed in the patient. The patient's family reported that he had been having gastrointestinal bleeding 4 days before admission, and he was suspected of having diabetes.

At admission, a physical examination indicated jaundice, hepatosplenomegaly, and ascites. A neurologic examination indicated that the patient was conscious, but disoriented, and that his vital signs were stable. The patient had a temperature of 38.9°C, a pulse rate of 130 beats/min, and blood pressure of 110/70 mm Hg. Other laboratory results were a leukocyte count of 2,900 cells/μL (70% neutrophils), a platelet count of 94,000/μL, a serum hemoglobin concentration of 13.20 g/dL, a glucose concentration of 195 mg/dL, a blood urea nitrogen level of 42 mg/dL, a creatinine level of 0.96 mg/dL; a serum bilirubin level of 3.01 mg/dL, an alanine aminotransferase level of 35 U/L, an aspartate aminotransferase level of 70 U/, a serum albumin level of 2.66 g/dL, and an increase in prothrombin time to 22 s.

Spontaneous bacterial peritonitis was suspected. Abdominal paracentesis was performed and produced a turbid milky fluid, with a protein level of 1600 mg/dL; 1,340 cells/μL (90% neutrophils), a lactate dehydrogenase level of 221 U/L, and an amylase level of 34 U/L. Samples of blood and ascitic fluid were inoculated into aerobic and anaerobic blood culture bottles. Gram staining was performed and no organisms were observed.

Treatment with intravenous ceftriaxone (2g/day) was started after a diagnosis of spontaneous bacterial peritonitis associated with liver cirrhosis was made. After 48 h of incubation, cultures of blood and ascetic fluid were plated onto sheep blood agar and chocolate agar and incubated at 35°C in an atmosphere of 5% $CO_2$. After 24 h of incubation, cultures showed growth of α-hemolytic streptococci.

An API Strep Test (bioMérieux, Marcy l'Etoile, France) identified the isolate as *S. pneumoniae* (probability 58.7%) or *S. suis* (probability 20.7%). However, these 2 probability values are unacceptable identification confidence levels. Therefore, the species and serotype were identified by sequence analysis of a 16S rRNA gene and a coagglutination test as described (*4*,*5*). The isolate was identified as *S. suis* serotype 21.

The infection was considered resolved when all signs and symptoms of infection disappeared, a polymorphonuclear cell count in ascitic fluid decreased to <250 cells/mL, and ascitic fluid cultures were negative for bacteria. Antimicrobial drug therapy was given for 48 h after resolution of the infection. The patient denied any recent occupational or occasional contact with swine or other animals, and he had no history of eating raw or undercooked pork.

A biochemically and antigenically atypical strain was isolated from the patient with peritonitis. A reference strain of serotype 21 and most other strains of this serotype had been isolated from tonsils of healthy pigs (*6*). However, 16 strains had also been isolated from sick pigs during 2008–2011 in Canada (*7*). These findings indicate that this serotype is potentially virulent. Most strains, including the strain from the patient reported, are usually not identified as *S. suis* by rapid multitest identification systems (*6*).

There are only 2 reports of *S. suis* being isolated from humans in Latin America; these reports were also from Argentina (*8*,*9*). Because swine production in Argentina is a smaller industry than in other Latin American countries, the higher rate of *S. suis* isolation rate is probably the consequence of good surveillance systems and awareness of the pathogen by local diagnostic laboratories.

The patient did not have any contact with swine, pork-derived products, or raw/undercooked beef. A patient infected with *S. suis* might be unaware or have no recollection of exposure to animals. Latent infection, with reactivation many years later, has been reported (*10*). *S. suis* might become an opportunistic pathogen in persons who are stressed or immunodeficient. This pathogen has also been increasingly isolated from mammals other than pigs and from the environment. The patient in this study had a history of alcohol consumption, which is a reported risk factor for this infection (*3*).

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# Cutaneous Leishmaniasis Caused by *Leishmania killicki*, Algeria

**To the Editor:** Cutaneous leishmaniasis (CL) is a widespread and resurging vector-borne disease caused by a protozoan parasite belonging to genus *Leishmania* (*1*). After Afghanistan, Algeria is the second largest focus of CL in the world. Although CL is a serious public health problem in Algeria, few data are available from this country.

During 2004–2008, an average of ≈44,050 CL cases were reported per year, and the estimated annual incidence ranged from 123,300 to 202,600 cases. Two main forms of CL have been described for more than a century in Algeria, the zoonotic, caused by *L. major* and the sporadic, caused by *L. infantum*. Since 2004, 11 strains belonging to the *L. tropica* complex, including *L. killicki* (*2*), were identified in 1 focus in the northern part of the Sahara (*3*) and in 2 foci in the northeastern Algeria (*4*,*5*). We report here a recent outbreak of CL, including infection with *L. killicki* strains, in the Tipaza area of northern Algeria.

Patients who sought treatment at Hajout hospital in Hajout, Algeria (a community of ≈51,000 persons), from January 2010 through April 2013 with cutaneous lesions consistent with leishmaniasis, underwent clinical examination. For each patient (146 total), we collected epidemiologic data (geographic origin, traveling history, especially to other leishmaniasis-endemic areas) and clinical data (number and size of lesions and clinical forms). Informed consent was obtained from all patients or their legal guardians. A particular characteristic of the infections was the unusual duration of some episodes, one of which persisted for >4 years, which is compatible with leishmaniasis recidivans (*6*).

Microbiological data were obtained as follows. Tissue samples, obtained by scraping the internal border of skin lesions from patients, were smeared onto a glass slide, fixed with methanol, stained with Giemsa, and examined by microscopy. Slides showing *Leishmania* amastigote forms were then processed further for molecular analyses. The immersion oil used to examine each slide was wiped off the smear with tissue paper, and then the dry smear was scraped from its slide by using a sterile scalpel. DNA extraction from smear scrapings was performed with the NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany). Species identification was performed by amplifying the topoisomerase II gene, followed by DNA sequencing (*7*).

In total, 60 patients exhibited *Leishmania*-positive cutaneous lesions as determined by microscopy. The topoisomerase II gene was successfully amplified and sequenced from samples from 38 patients. *Leishmania* species were identified by comparing sequences with those of the reference strains *L. infantum* MHOM/FR/78/LEM75, *L. killicki* MHOM/TN/80/LEM163, and *L. major* MHOM/MA/81/LEM265 (*7*). *L. infantum* was identified in 36 cases and *L. killicki* in 2 cases (Figure). No *L. major* isolates were found in this series.

The low proportion of *L. killicki* strains was similar to that found recently in the Annaba focus in northeastern Algeria (*5*). However, the observation of a new focus of CL and *L. killicki* as etiologic agent may indicate a modification of the epidemiology of CL in Algeria. This focus, located far from other previously described areas where the *L. tropica* complex is endemic, may reflect geographic spread of this complex in Algeria.

The results of this study can be placed in a larger framework as well. Since 2004, strains in the *L. tropica* complex have been increasingly reported as responsible for CL

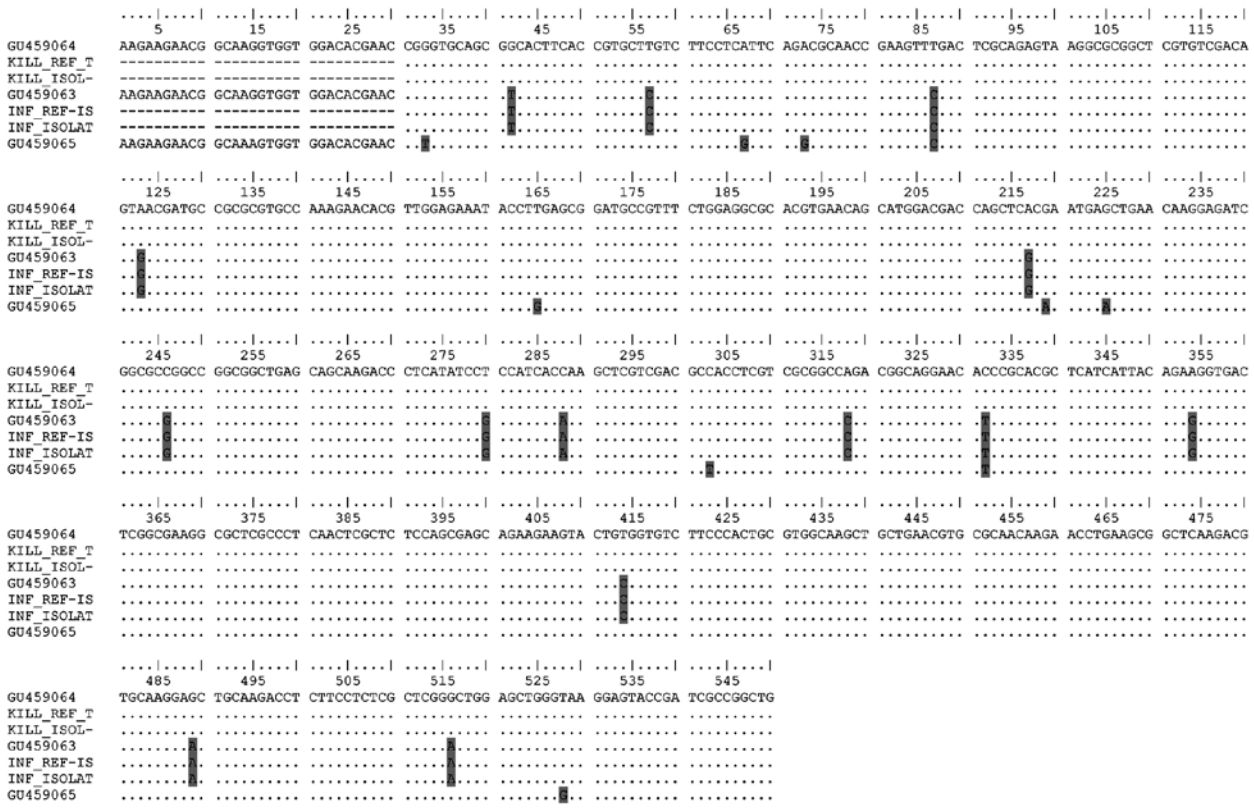

Figure. Alignment of topoisomerase II nucleotide sequences of *Leishmania killicki*, *L. infantum*, and *L. major*. Point mutations discriminating *Leishmania* species are outlined on a gray background. The references strains are GU459063: *L. infantum* MHOM/FR/78/LEM75; GU459064: *L. killicki* MHOM/TN/80/LEM163; GU459065: *L. major* MHOM/MA/81/LEM265 ; KILL_REF_T: *L. killicki* and INF_REF-IS: *L. infantum*, strains genotyped by the *Leishmania* National Reference Center, Montpellier, France. The isolates are: KILL_ISOL-: *L. killicki* (n = 2); INF_ISOLAT: *L. infantum* (n = 36).

in Mediterranean countries, in the Near East and Middle East (*2*), possibly in relation to changes in environmental conditions. Urbanization and/or climatic changes that have occurred in recent years could have played a role in the spread of the disease. The cases reported here were observed in urban areas, which suggests transmission according to an anthroponotic mode.

Each species responsible for CL has its own epidemiologic pattern. Clinicians must be aware of the specificity of leishmaniases that may be encountered in North African countries. *L. tropica* complex lesions heal spontaneously over a period of 12 months or more, a duration longer than for *L. major* infections (*8*). *L. tropica* infections are also less responsive to treatment compared to infections with other Old World *Leishmania* species. In addition, *L. tropica* may cause leishmaniasis recidivans. This type of CL, appearing often years after the initial infection showed signs of complete resolution, manifests as papules that transform slowly into a spreading granuloma resembling lupus vulgaris (*6*). *L. tropica* can also produce visceral infections on rare occasions, resulting in unexplained systemic illness, including classic symptoms of visceral leishmaniasis, in persons returning from areas where this *Leishmania* complex is endemic (*9*).

Other epidemiologic studies are required to detect additional foci, including those of the *L. tropica* complex, that may coexist with those of *L. infantum* and *L. major* in Algeria. Travelers to North Africa should also be informed about the existence of this spreading disease (*10*).

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# Rift Valley Fever in Kedougou, Southeastern Senegal, 2012

**To the Editor:** Rift Valley fever (RVF) is an acute, febrile, viral disease caused by Rift Valley fever virus (RVFV), a phlebovirus of the family *Bunyaviridae* that is endemic to sub-Saharan Africa. RVF mortality and abortion rates among young domesticated ruminants and pregnant females are high.

In humans, clinical manifestations range from mild to severe syndromes, which can include neurologic, hemorrhagic, and hepatic features and retinitis, and which sometimes result in death (*1*). Diagnosis of RVF is challenging for clinicians because clinical manifestations are not specific (*2*). Heavy rainfall and flooding create conditions for emergence of RVF vectors (*Aedes* and *Culex* spp. mosquitoes), and dispersion of this disease into new areas is linked to migration of infected livestock, wildlife, or mosquitoes.

Since 1987, when the Diama dam was built, RVF outbreaks in Mauritania have been reported regularly (*3*). In Kedougou, southeastern Senegal, RVFV was isolated 4 times from *Ae. dalzieli* mosquitoes and once from a person with a mild case of RVF (*4*). We report results of a field investigation and laboratory findings for a human case of RVF detected by surveillance of acute febrile illnesses in Kedougou.

On October 16, 2012, a 27-year-old man (school teacher) who lived and worked in Baya village in the Kedougou region of Senegal (12°27′50″N, 12°28′6″W) visited the Kedougou military health post because of high fever, chills, headache, back pain, myalgia, and arthralgia that started on October 14. He reported regular contact with domesticated animals (cows, sheep, and goats) during farming.

A thick blood smear for the patient showed a positive result for malaria, and specific treatment was given. As part of surveillance for acute febrile illnesses, blood samples from the patient were tested for IgM against RVF, chikungunya, dengue, West Nile, yellow fever, Zika, and Crimean-Congo hemorrhagic fever viruses; and for viral RNA and virus (*5*,*6*). All test results for IgM against the 7 viruses were negative

RVFV was isolated from newborn mice that were intracerebrally inoculated with a blood sample from the patient. Viral RNA was detected by reverse transcription PCR in serum from the patient. Phylogenetic analysis of the partial nonstructural protein gene on the small RNA segment showed that the RVFV isolate was closely related to a strain that had circulated in Mauritania in 2012 (Figure).

An epidemiologic field investigation was conducted to assess the extent of RVFV circulation. During this investigation, the case-patient provided an additional blood sample. In addition, 115 contacts of the case-patient, including primary school students, friends, family members and neighbors (median age 12 years, range 6–75 years; female:male sex ratio 1.6) were also sampled and questioned to identify asymptomatic and benign cases. A total of 218 samples from patients attending the nearest health posts in Ibel and Thiokoye villages during October 2012 were also tested during surveillance of acute febrile illnesses.

All 334 samples were negative for RVFV RNA and IgM and IgG against RVFV except for samples from 3 patients, including the case-patient, which were positive for RVFV-specific IgG and malaria parasites. The 2 other patients were a 32-year-old tradesman and a 20-year-old housewife sampled during surveillance of acute febrile illnesses in Kedougou and Bandafassi, which is 30 km from Baya (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/3/13-1174-Techapp1.pdf). No RVFV RNA was detected

from 519 mosquito pools sampled in the Kedougou region during October 2012, although these pools included 7 species previously found associated with RVFV and which represented 26.6 % of the pools.

The patient reported no travel outside Kedougou in the 2-year period before his illness. Because no evidence of recent RVFV circulation among humans and mosquitoes was found, we believe that the patient was infected by contact with an animal imported from Mauritania. This hypothesis is based on reports by farmers from neighboring villages (Baya, Ibel, Thiokoye, and Dondol) of the presence of ruminants imported from Mauritania in the market in Thiokoye village and of deaths and abortions among sheep and goats in their villages during October–November 2012. However, no animals were sampled during the investigation.

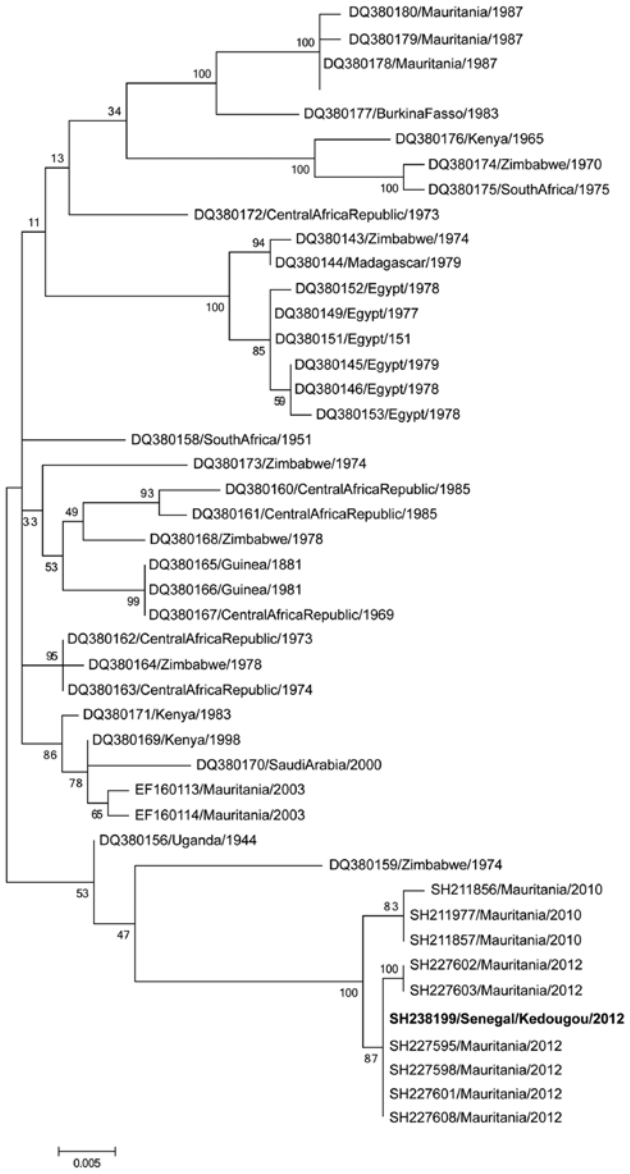


Figure. Phylogenetic tree of a 581-bp sequence of the nonstructural protein gene on the small RNA segment of Rift Valley fever viruses. **Boldface** indicates strain isolated in this study. Bootstrap values are indicated along branches. Scale bar indicates nucleotide substitutions per site.

There is an abundance of competent vectors for RVFV in Kedougou (*4*). In addition, there are massive human migrations resulting from gold mining and regular importation of animals from RVF-endemic regions of western Africa. Thus, an integrated human and animal surveillance system should be implemented or reinforced to avoid large-scale RVF outbreaks in Kedougou.


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# Concomitant Multidrug-Resistant Pulmonary Tuberculosis and Susceptible Tuberculous Meningitis

**To the Editor:** In 2012, a 34-year-old HIV-seronegative man was hospitalized after several months of cough, fever, night sweats, 10-kg weight loss, and, in the past month, severe headache. The patient was born in Romania and had lived in France for 2 years. He had a history of pulmonary tuberculosis (TB) for which treatment was started in Romania in 2006 and 2008, but he did not complete treatment. The treatment he received in Romania was unknown.

At hospital admission, the patient had a fever of 39°C, stiff neck, and swollen cervical and axillary lymph nodes. A chest radiograph showed multiple cavities and nodular opacities in both superior lobes. Sputum auramine staining indicated that acid-fast bacilli was positive, which supported the diagnosis of pulmonary TB. Examination of cerebrospinal fluid (CSF) revealed hypoglycorrachia (0.95 mmol/L, concentration ratio CSF/blood: 0.2 [reference range 0.5–0.75]), hyperproteinorrachia (1.3 g/L [reference range 0.2–0.4 g/L]), erythrocyte count 2,000 μL (reference value <10 μL), and leukocyte count 150 μL (reference value <10 μL). Auramine staining showed no acid-fast bacilli in CSF. Standard antituberculous therapy with rifampin (RIF), isoniazid (INH), pyrazinamide, and ethambutol was started.

Genomic amplification–based assay (Xpert MTB/RIF; Cepheid, Maurens-Scopont, France), performed on sputum, confirmed the presence of the *Mycobacterium tuberculosis* genome and detected resistance to RIF (Table). The line probe assay GenoType MTBDR*plus* (Hain Lifescience, Bandol, France) performed on sputum showed a positive signal for all wild-type sequences and for *rpoB* (S531L associated with RIF resistance) and *katG* (S315T associated with INH resistance) mutations, suggesting the presence of mixed susceptible and resistant *M. tuberculosis*. Second-line treatment was started: moxifloxacin, amikacin, ethionamide, para-aminosalicylic acid, cycloserine and linezolid. The presence of mixed *M. tuberculosis* organisms in lungs was confirmed with culture methods and by phenotypic drug susceptibility testing (DST) that showed 1% resistant mutant to RIF and INH (proportion method) (*1*). The isolate was considered resistant to RIF and INH and was thus categorized as multidrug resistant (MDR). DST also showed that the sputum isolate had an elevated proportion of ofloxacin-resistant mutants (2 mg/L, 0.02% resistant mutants). Although no mutation in *gyrA* or *gyrB* was detected on colonies grown in the absence of fluoroquinolone, a *gyrB* N538D mutation was identified on colonies grown on ofloxacin medium. On the basis of the DST results, the treatment was changed to ethambutol, pyrazinamide, amikacin, moxifloxacin, para-aminosalicylic acid, and linezolid. CSF culture was eventually positive for *M. tuberculosis* on Lowenstein-Jensen medium after 30 days. DST performed on the CSF isolate showed a drug-sensitive phenotype; thus, RIF was reintroduced in addition to the other antimicrobial drugs. After 3 months, although the patient had improved, he left the hospital against medical advice without providing follow-up contact information.

Mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) analysis was conducted on culture of the sputum and CSF sample and on colonies grown on INH, RIF, and ofloxacin–containing medium, as described (*2*) (Table). This analysis showed different genotypes for the lung MDR and the CSF-susceptible isolates. None of the MIRU-VNTR patterns

Table. Localization, drug-susceptibility results, and MIRU-VNTR genotypes of *Mycobacterium tuberculosis* strains recovered from a man with both pulmonary tuberculosis and tuberculous meningitis, France, 2012*

| Tissue | MIRU-VNTR genotype 1<br>222213222234225153333622 | MIRU-VNTR genotype 2<br>224243122234225143335622 |
|---|---|---|
| Lung | Isoniazid and rifampin resistant, fluoroquinolone susceptible, *rpoB* S531L, *katG* S315T, no mutations in *gyrA* and *gyrB* | Pan-susceptible except to fluoroquinolones, no mutations in *rpoB*, *katG,* and *gyrA*; *gyrB* N538D |
| Cerebrospinal fluid | | Pan-susceptible, no mutations in *rpoB*, *katG*, *gyrA*, and *gyrB* |

*MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat.

were linked to known lineages. The mutation rate of MIRU-VNTR has been evaluated at 2.7 ´ 10–3/loci/year (*3*). Rather than natural evolution of 1 strain, the 5-locus difference observed for the MIRU-VNTR genotypes is probably attributable to infection with 2 *M. tuberculosis* strains. Surprisingly, MIRU-VNTR patterns were identical for the *gyrB* mutated lung isolate and to the antimicrobial drug–susceptible isolate obtained from CSF. Also, the *gyrB* mutated lung isolate was susceptible to other antimicrobial drugs, whereas the lung MDR isolate did not harbor any *gyrB* mutation. Taken together, both genotype and DST heterogeneity were shown (Table). None of the drug-resistant clones were isolated in CSF.

This heterogenous infection raises 2 hypotheses: the patient was infected with both the MDR and the non-MDR strains or with 2 non-MDR strains and acquired additional drug resistance in the lung during treatment. Clonal differences among *M. tuberculosis* culture isolates obtained from pulmonary and CSF samples indicate compartmentalization (*4*). Heterogeneity in *M. tuberculosis* isolates already has been reported either for genotypes or for drug susceptibility (*5*–*7*). However, combination of genotype and resistance heterogeneity is a diagnostic and therapeutic challenge (*8*). In our laboratory, the line probe assay performed directly on sputum detected the 1% INH- and RIF-resistant mycobacteria, although a previous report suggest that this assay could not detect <5% resistant mycobacteria (*9*). The antimicrobial drug–resistant clones were isolated from only lung. The higher bacillary population in lungs than in central nervous system may account for an increased selection of resistant mutants in lungs. This finding raises the question of whether DST obtained from lung isolates should be used for establishing TB treatment at extrapulmonary localizations, especially in patients with meningitis, for whom effective treatment is an emergency. Because first-line treatments are more effective, we wonder whether treatment for MDR TB patients who have secondarily acquired resistance and both lung and central nervous system TB should target MDR and drug-susceptible TB until the CSF strain is proven to also be MDR.

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# *Anaplasma phagocytophilum* Antibodies in Humans, Japan, 2010–2011

**To the Editor:** Human granulocytic anaplasmosis (HGA) is an emerging tick-borne infectious disease caused by *Anaplasma phagocytophilum*, an obligatory intracellular bacterium (*1*). Recently, 2 cases of HGA were identified by a retrospective study in Japan (*2*). For serodiagnosis of HGA, *A. phagocytophilum* propagated in HL60 cells is usually used as an antigen, especially by indirect immunofluorescent assay (IFA) (*3*). However, the serum from these 2 patients in Japan reacted with antigens of *A. phagocytophilum* cultured in THP-1 cells rather than in HL60 cells in IFA (*2*). In *A. phagocytophilum*, a *p44/msp2* multigene family encoding multiple 44-kDa immunodominant major outer membrane protein species (so-called P44) exists on the genome, and these multigenes are similar, but not identical, to each other, and the bacterium generates antigenic variations because of gene conversion (*4*). The previous studies showed that *A. phagocytophilum* expresses predominantly 2 species of *p44*/*msp2* transcripts in THP-1 cells, but it produces the variation of P44 protein species in HL60 cells (*2*,*5*). This finding strongly suggested that *A. phagocytophilum* grown in THP-1 cells differs serologically from that in HL60 cells. Our serologic analysis found 4 recent cases of HGA in Japan by using infected THP-1 and HL60 cells as antigens, and some P44 immunoreactive protein species of *A. phagocytophilum* that were associated with the respective cell line cultures, binding to antibodies from the 4 patients' serum, also were identified.

In 2010 and 2011, nine patients in Shizuoka Prefecture, Japan, who had rickettsiosis-like symptoms, were suspected to have Japanese spotted fever or scrub typhus, but they were serologically negative by IFA. Therefore, IFA for HGA was conducted. In 4 of the patients, antibodies to *A. phagocytophilum* were detected in serum by using *A. phagocytophilum* cultured in THP-1 and HL60 cells as antigens (Table). In IFA tests for HGA, IgM and/or IgG from the patients' serum samples reacted with *A. phagocytophilum* cultured in THP-1, HL60, or both, and the seroconversions were observed in convalescent-phase serum from all patients. The clinical manifestation and laboratory findings for the 4 patients are summarized in the online Technical Appendix Table, (wwwnc.cdc.gov/EID/article/20/3/13-1337-Techapp1.pdf). Western blot analysis further confirmed the specific reaction to P44 protein antigens (P44s) of *A. phagocytophilum* cultured in THP-1 and HL60 and to recombinant P44–1 protein (rP44–1) in the serum samples (online Technical Appendix Figures 1 and 2), supporting the IFA results in the Table.

To identify P44 immunodominant protein species binding to antibodies from the patients' serum, we selected P44–47E and P44–60 proteins that are dominantly expressed by *A. phagocytophilum* propagated in THP-1 cells (*2*) and P44–18ES protein that frequently predominates by *A. phagocytophilum* cultured in HL60 cells (*6*) as representatives for the preparation of recombinant proteins. The central hypervariable regions of the respective P44 proteins (online Technical Appendix Figure 3) were produced as recombinant proteins in vitro by insect cell–free protein synthesis system (Transdirect Insect Cell Kit; Shimadzu Co., Kyoto, Japan) (*7*) to avoid the strong nonspecific reaction with human serum that occurs in the *Escherichia coli* expression system. In Western blot analyses using these 3 recombinant P44 proteins (rP44–60 and rP44–47E for THP-1 and rP44–18ES for HL60) as antigens, most of the serum from the patients was reactive with *A. phagocytophilum* cultured in THP-1 cells in IFA bound to either rP44–60 or rP44–47E, whereas the

Table. Immunofluorescence antibody titers to *Anaplasma phagocytophilum* in serum from 4 patients with human granulocytic anaplasmosis and reactive rP44 protein species, Japan, 2010–2011*

| | | Antigen | | | |
|---|---|---|---|---|---|
| | | *A. phagocytophilum* propagated in THP-1 cells (rP44 species) | | *A. phagocytophilum* propagated in HL60 cells (rP44 species) | |
| Patient no. | Time after illness onset, d | IgM | IgG | IgM | IgG |
| 1 | 1 | 80 (r60) | <20 | 80 (r18ES) | <20 |
| | 15 | 160 (r60) | <20 | 160 (r18ES) | <20 |
| | 30 | 320 (r60) | 20 (r60) | 320 (r18ES) | <20 |
| 2 | 13 | 40 | 40 (r47E) | <20 | 20 |
| 3 | 3 | 40 | 80 (r60) | <20 | 20 (r18ES) |
| | 7 | 40 | 80 (r60) | <20 | 20 (r18ES) |
| | 24 | 80 (r60) | 160 (r60) | <20 | 40 (r18ES) |
| 4 | 4 | 160 (r47E) | 40 | <20 | <20 |
| | 15 | 160 (r47E) | 80 | <20 | <20 |

*Three recombinant P44 (rP44) protein species (r18ES, r47E, r60) were prepared and either one bound to antibodies in each serum sample from 4 patients in Western blot analyses (online Technical Appendix Figure 4, wwwnc.cdc.gov/EID/article/20/3/13-1337-Techapp1.pdf). r18ES represents rP44–18ES immunoreactive outer membrane protein that is known to predominate in *A. phagocytophilum* cultured in HL60 cells (*6*). r47E and r60 show rP44–47E and rP44–60 proteins, respectively, that are dominantly transcribed in *A. phagocytophilum* propagated in THP-1 cells (*2*).

patients' serum reactive with *A. phagocytophilum* cultured in HL60 cells in IFA bound to rP44–18ES (online Technical Appendix Figure 4; Table). This finding strongly supports the results of IFA and Western blot analyses with the infected THP-1 and HL60 cells.

In Japan, rickettsioses such as Japanese spotted fever and scrub typhus, caused by *Rickettsia japonica* and *Orientia tsutsugamushi*, respectively, occur frequently. However, fever of unknown cause and rickettsiosis-like symptoms still occur in some patients. Detection of *A. phagocytophilum* in ticks was first reported in 2005 in central Japan (*8*). Since then, DNA of *A. phagocytophilum* has been detected in ticks inhabiting several places of Japan (*9*,*10*). However, little was known about human infection with *A. phagocytophilum* for many years, probably because of the poor selection of the culture cell line used as infected cell antigens for serodiagnosis. Our previous study first documented HGA in Japan and recommended that *A. phagocytophilum* propagated in THP-1 and in HL60 cells be used as antigens to avoid misdiagnosing cases of HGA. Our current study demonstrates the presence of specific antibodies against the central hypervariable regions of P44–47E, P44–60, or P44–18ES proteins that predominate in infected THP-1 or HL60 cells, probably being suitable as protein antigens for serodiagnosis of HGA. The rP44–1 protein whose recombinant plasmid had previously been constructed for *E. coli* expression system may be available as well. Thus, our study provides substantial information about the usefulness of suitable P44 immunoreactive protein species of *A. phagocytophilum* as antigens for serodiagnosis of HGA.

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# *Cyclospora* spp. in Drills, Bioko Island, Equatorial Guinea

**To the Editor:** More than a decade has passed since major outbreaks of *Cyclospora cayetanensis* infection in the United States and Canada drew attention to this newly emerging infection (*1*,*2*). Awareness of these infections was highlighted again by large outbreaks in the summer of 2013 (*3*). However, many questions remain unanswered regarding this organism, including aspects of its life cycle, geographic distribution, and range of related species.

In 1999, three new distinct *Cyclospora* species noted for their close similarity with *C. cayetanensis* from humans were isolated from monkeys in Ethiopia (*4*). A survey of primates in Kenya increased awareness of the extended distribution of these 3 species in eastern Africa and provided confirmation of their marked host specificity, even where the ranges of host species overlapped (*5*). Most recently, *C. colobi*–like organisms were identified in snub-nosed golden colobus monkeys in northwestern China (*6*). We report the characterization of *Cyclospora* spp. recovered from drills (*Mandrillus leucophaeus poensis*) on Bioko Island, Equatorial Guinea.

During January–February 2011 and 2012, fecal samples from free-ranging animals were collected and placed in 10% formalin (2011) or potassium dichromate (2012). Because samples were collected opportunistically from unidentified animals of undetermined age and sex, whether any samples were collected from the same animals in either year was not known. Fecal samples were concentrated by using the formyl ethyl acetate method, and sediment was examined by using fluorescent microscopy to detect oocysts (*4*).

Three (9%) of 26 samples from 2011 and 8 (31%) of 25 samples from 2012 were positive for *Cyclospora* oocysts that were spherical, measured 8–10 μm in diameter, and showed autofluorescence. The oocysts collected in potassium dichromate had sporulated by the time of examination, which facilitated and confirmed identification as *Cyclospora* spp. Representative samples from 4 animals in the second collection were submitted for molecular analysis.

The entire 18S rRNA gene (1,796 bp) was obtained from 2 DNA fragments amplified by PCR from DNA extracted from 3 fecal specimens by using procedures and primers for genetic analysis of coccidian parasites (*4*,*7*,*8*). Six distinct full-length 18S rRNA sequences were obtained and compared with sequences in GenBank.

Although our sequences showed high similarity with 18S rRNA genes for all *Cyclospora* species, the sequences were most similar to the *C. papionis* 18S rRNA gene (GenBank accession no. AF111187), even though 3 T → C transitions at nucleotides positions 680, 1054, and 1694 were observed. Analyses of these 6 sequences showed intravariation caused mainly by T→C and A→G transitions. Further studies on different species should be performed to verify whether this is a common feature in *Cyclospora* spp. 18S rRNA genes.

This report extends our knowledge of the range of *Cyclospora* spp. in monkeys to include western Africa and their host range to include an additional distinct primate species. Results of molecular analysis indicate that this *Cyclospora* sp. isolate from drills on Bioko Island is most similar to *C. papionis* from baboons in eastern Africa, an observation that is unexpected and somewhat difficult to explain. Previous studies have suggested that different primate hosts harbor distinctly different *Cyclospora* species (*4*–*6*). Baboons are not found on Bioko Island or in mainland Equatorial Guinea near Bioko Island and are allopatric with drills on the mainland. In addition, drills on Bioko Island have been separated from contact with drills on the mainland for 10,000–12,000 years (*9*), further isolating the ecology of this host–parasite relationship and confusing how *C. papionis* was established in drills on Bioko Island.

Drills are now considered to have closer phylogenetic affinity with mangabeys (*Cercocebus* spp.) than with baboons (*10*), although the phylogeny of these primates is not completely resolved. This finding further confuses an explanation of why the parasite isolated from drills would be similar to that recovered from baboons. It could be speculated that *C. papionis* arrived on Bioko Island from the mainland through some third host, such as collared (red-capped) mangabeys (*C. torquatus*), which has close phylogenetic relationships and overlapping ranges with drills and baboons. Any such explanation would mean that *Cyclospora* spp. infected drills before Bioko Island and the mainland separated.

Another possibility is that *Cyclospora* spp. exhibit host–niche specificity. Colobus monkeys, the host for *C. colobi*, are arboreal folivores, many of which consume relatively difficult-to-digest foods and have large specialized guts. Vervets, hosts for *C. cercopitheci*, are also arboreal, but have a frugivorous–insectivorous diet and consume little leaf matter. Baboons and drills, hosts for *C. papionis*, are predominantly terrestrial and have generalist–omnivorous diets and unspecialized guts. These different ecologic and physiologic differences among the 3 species may affect the observed *Cyclospora* spp. host specificity.

Observations in the present study extend our knowledge of the geographic and host range for cyclosporiasis. However, these observations leave several unanswered questions about our understanding of the parasite in nonhuman primates; the evolutionary relationship between human

*C. cayetanensis* and these closely related species in monkeys; what additional monkey host species, especially on Bioko Island, may harbor *Cyclospora* spp.; and what other as yet unrecognized species of *Cyclospora* may be infecting primates.

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# Novel Cetacean Morbillivirus in Guiana Dolphin, Brazil

**To the Editor:** Since 1987, morbillivirus (family *Paramyxoviridae*, genus *Morbillivirus*) outbreaks among pinnipeds and cetaceans in the Northern Hemisphere have caused high rates of death (*1*,*2*). Two morbillivirus species are known to affect aquatic animals: *Phocine distemper virus* (PDV) and *Cetacean morbillivirus* (CeMV). PDV has been isolated from pinnipeds, and 3 strains of CeMV (porpoise morbillivirus [PMV], dolphin morbillivirus [DMV], and pilot whale morbillivirus [PWMV]) have been isolated from dolphins and whales (*3*,*4*).

Serologic surveys indicate that morbilliviruses infect marine mammals worldwide (*5*); however, only 1 fatal case in a bottlenose dolphin (*Tursiops truncatus*) has been confirmed in the Southern Hemisphere (in the southwestern Pacific Ocean) (*6*). Positive DMV-specific antibody titers in 3 Fraser's dolphins (*Lagenodelphis hosei*) stranded off Brazil and Argentina in 1999 indicate the exposure of South Atlantic cetaceans to morbillivirus (*7*). We report a case of lethal morbillivirus infection in a Guiana dolphin (*Sotalia guianensis*), a coastal marine and estuarine species that occurs off the Atlantic Coast of South and Central America.

A female Guiana dolphin calf (108 cm in total body length) (*8*) was found stranded dead in Guriri (18°44′S; 39°44′W), São Mateus, Espírito Santo State, Brazil, on November 30, 2010; the dead calf was severely emaciated. Postmortem examination of the animal showed multifocal ulcers in the oral mucosa and genital slit, diffusely dark red and edematous lungs, and congested and edematous brain. Samples of selected tissues were collected, fixed in buffered formalin, and processed according to routine histopathologic methods. By microscopy, the most noteworthy lesions included marked lymphoplasmacytic and neutrophilic meningoencephalitis, optic nerve perineuritis, and hypophysitis. Lungs showed moderate acute diffuse lymphoplasmacytic and neutrophilic interstitial pneumonia; severe multicentric lymphoid depletion and multifocal necrotizing hepatitis were also observed.

Immunohistochemical analysis was performed by using CDV-NP MAb (VMRD, Inc., Pullman, WA, USA), a monoclonal antibody against the nucleoprotein antigen of canine distemper virus that cross-reacts with cetacean morbilliviruses (*9*). Known positive and negative control tissues and test sections with omitted first-layer antibody were included. Viral antigen was detected in neurons in the brain,

bronchiolar epithelium and macrophages in the lungs, bile duct epithelium in the liver, and macrophages and lymphocytes in lymph nodes.

We extracted RNA from frozen lung samples by using TRIzol Reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions and amplified a 374-bp conserved fragment of the phosphoprotein (P) gene by reverse transcription PCR. The following *Morbillivirus* spp.–specific primers were used for PCR: 5′-ATGTTTATGATCACAGCGGT-3′ (forward) and 5′-ATTGGGTTGCACCACTTGTC-3′ (reverse) (*3*). MEGA5 (http://megasoftware.net/) was used to construct a neighbor-joining phylogenic tree based on the sequenced amplicon from this study (GenBank accession no. KF711855) and 12 other GenBank sequences that represent the 6 morbillivirus species already described in the literature. The analysis placed the Guiana dolphin strain at the CeMV clade, but segregated it from the already described dolphin morbillivirus strains PMV, DMV, and PWMV (Figure). The sample shared 79.8% nt and 58.4% aa identity with PMV, 78.7% nt and 56.6% aa identity with DMV, and 78.7% nt and 57.1% aa identity with PWMV. Within the *Morbillivirus* spp., PDV shared the lowest sequence identity (51.1% nt and 26.8% aa).

In summary, sequence analysis of the morbillivirus from the dead Guiana dolphin suggests that the virus is a novel strain of the CeMV species; this conclusion is supported by phylogenic analysis and geographic distribution of the virus and by its distinct host. Emaciation, marked lymphoid depletion, interstitial pneumonia, and meningoencephalitis are common findings in morbillivirus-infected animals (*1*,*2*). Together with antigenic and genomic evidence, our findings indicate that morbillivirus infection is extant in Guiana dolphins in the waters off Brazil.

Morbillivirus outbreaks have caused a high number of deaths among pinnipeds and cetaceans and are a major risk to previously unexposed nonimmune populations of aquatic mammals (*1*,*2*). A high number of morbillivirus-related deaths have not yet been reported among aquatic mammals in the waters off Brazil, but our findings shows that Guiana dolphin calves are susceptible to infection. Subclinical morbillivirus infection with immune suppression has been reported in bottlenose dolphins in Florida (*10*). It is unknown whether subclinical infection occurs in this host population or whether the virus has undergone species-adaptive changes, as proposed for PWMV (*4*). The sequence data from our study suggest that the virus from the Guiana dolphin calf is the fourth member of the CeMV group and is closer to the root of the CeMV clade than to that of DMV, PMV, or PWMV. Further studies are required to determine the epidemiology of morbillivirus infection in this and other cetacean species and to assess the risk for epizootic outbreaks among South Atlantic cetaceans.

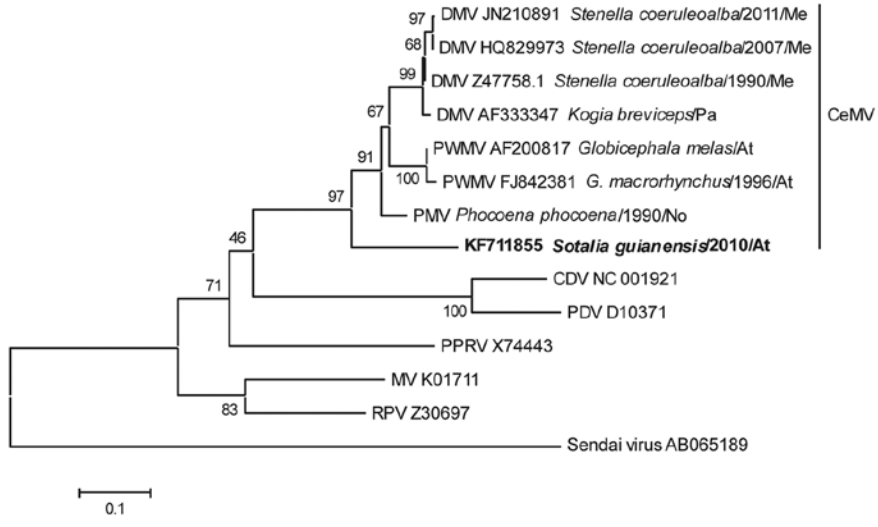


Figure. Phylogenetic tree of a 374-bp conserved region from the phosphoprotein gene of a cetacean morbillivirus isolated from a Guiana dolphin (in boldface; GenBank accession no. KF711855) and 12 other previously described morbilliviruses. Sendai virus was added as an outgroup member. Sequences were aligned and a neighbor-joining tree with 1,000 bootstrap replications was generated by using MEGA5 (http://megasoftware.net/). For comparison, recognized viruses of the *Morbillivirus* spp. (*Measles virus* [MV], *Rinderpest virus* [RPV], *Peste-des-petits ruminants virus* [PPRV], *Canine distemper virus* [CDV], and *Phocine distemper virus* [PDV]) were included, as were the 3 *Cetacean morbillivirus* (CeMV) strains: porpoise morbillivirus (PMV), dolphin morbillivirus (DMV), and pilot whale morbillivirus (PWMV). Sequence names are followed by species of cetacean, year of stranding (when available), and the abbreviation for the geographic area. Me, Mediterranean Coast; Pa, Pacific Ocean; At, Atlantic Ocean; No, North Sea. The sequence for PMV strain *Phocoena phocoena*, is from Barrett et al. (*3*). The scale bar indicates nucleotide substitutions per site.

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# In Memoriam: James Harlan Steele (1913–2013)

**Myron G. Schultz**

James Steele, DVM, MPH, passed away on November 10, 2013, in Houston; he was 100 years old. Jim Steele was an extraordinary man. All of the dimensions of his life were on a grand scale. He was larger than life in so many ways; his vision, his leadership, his accomplishments in public health, his worldwide friendships, his mentorship of scores of young acolytes who came within his orbit, his extraordinary memory, his bear hugs, and his longevity were all manifestations of his boundless enthusiasm for life.

Dr Steele's professional career spanned more than 70 years. It began in 1938 when he worked in a brucellosis testing laboratory for the Michigan State Department of Agriculture while studying veterinary medicine at Michigan State University. Brucellosis developed in many of his veterinary colleagues, and he wanted to learn how the causative pathogen and other pathogens were transmitted from animals to humans. This was the beginning of his lifelong vocation of studying and controlling zoonotic diseases.

In 1941, Dr Steele received a doctorate of veterinary medicine from Michigan State University, and in 1942, he earned a master of public health degree from Harvard University. In 1943, he was commissioned as a sanitarian in the Public Health Service (PHS). He spent most of World War II in Puerto Rico and the Virgin Islands, where he coordinated milk and food sanitation programs, evaluated zoonotic threats to the islands, and conducted research on brucellosis, bovine tuberculosis, rabies, and Venezuelan equine encephalitis.

After the war, Dr Steele's encounter with Assistant Surgeon General Joseph Mountin, the legendary founder of the Communicable Disease Center (now named Centers for Disease Control and Prevention; CDC), changed his career. Dr Steele was fond of telling how Dr Mountin challenged him by asking, "What are you veterinarians going to do now that the war is over?" In response, Dr Steele described some of the known zoonotic diseases, and Dr Mountin asked questions about their prevalence and control. Dr Steele's main response was "We don't have any data, nor do we know how to control these zoonoses." In the end, Dr Mountin said, "Steele, it is quite apparent that we have a problem and a lot of ignorance—let us exploit it!" Thus, in 1945, Dr Steele produced a detailed report titled Veterinary Public Health, which outlined the risks posed by zoonotic diseases and the benefits of employing veterinarians for research and response efforts. Dr Mountin and Surgeon General Thomas Parran were impressed by the scope of the report, and in 1947, Dr Steele convinced the Surgeon General to establish a Veterinary Medical Officer category in the PHS. He entered this service category and became the PHS chief veterinary officer. When he retired from the PHS Commissioned Corps

James Harlan Steele

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DOI: http://dx.doi.org/10.3201/eid2003.IM2003

in 1971, Dr Steele was an Assistant Surgeon General, the first veterinarian to achieve this rank.

Dr Steele came to CDC in 1947, just after its beginning. There was no road map for the work he did—he was a pioneer, creating CDC's veterinary public health program. Much of his work was focused on rabies eradication. He and his team improved the existing vaccine, and he then worked toward eliminating the disease in dogs and cats in the United States and other countries. He also worked on other diseases that threatened humans and animals, including bovine tuberculosis and brucellosis, Q fever, psittacosis, salmonellosis and other food-borne diseases, and avian influenza.

Dr Steele also pioneered the integration of veterinary public health into the Pan American Health Organization (PAHO) and the World Health Organization (WHO). In 1950, he attended the first WHO Expert Committee meeting, and in 1965, he chaired the second meeting. These meetings brought together the most eminent experts in the world of zoonotic diseases and emphasized the need for international collaboration and common goals. Jim Steele worked closely with PAHO and WHO throughout his career.

Dr Steele enjoyed a good relationship with Dr Alexander Langmuir, who founded CDC's Epidemic Intelligence Service (EIS) training program in 1951. In 1953, Dr Langmuir asked Dr Steele to recruit veterinarians to work in all epidemiologic areas (animal and nonanimal diseases) of the EIS program. This was the beginning of a new sphere of opportunity for veterinarians in public health. Today, veterinarians are integrated into all areas of PHS activity.

When he retired from PHS in 1971, Dr Steele became a professor at the University of Texas School of Public Health. He was an active teacher, writer, and mentor. He compiled the CRC Handbook Series in Zoonoses, the first comprehensive collection addressing diseases shared by humans and animals. The book remains a staple of public health curricula throughout the world.

Dr Steele received numerous awards during his career. Among them are the American Public Health Association's Bronfman Prize, the American Veterinary Medical Association's International Veterinary Congress Prize, the Surgeon General's Medallion, the PAHO Abraham Horwitz Award for Excellence in Leadership in Inter-American Health, the OIE (World Organization for Animal Health) Medal of Merit, and many more. In addition, the University of Texas School of Public Health holds an annual James Steele Lecture, and a James H. Steele Veterinary Public Health Award is given annually at CDC's EIS Conference.

The message of the One Health Initiative is that human health and animal health are inextricably linked: we cannot have good public health unless we have good animal health, and we cannot have good animal health unless we have good public health. Jim Steele was a father of the One Health Initiative. He didn't merely profess this concept—he practiced it for 7 decades, and he taught it to younger generations of veterinarians. Jim Steele had an extraordinary capacity for mentoring younger health professionals and sustaining lifelong relationships.

Lewis Thomas, the physician–philosopher who wrote about so many aspects of life, said that the highest state of life is to be useful—to be engaged in purposeful activity with your fellow men. By this measure, Jim Steele was a rich man—not in material wealth, which is ephemeral—but in his relationships with other human beings, which are enduring. Jim Steele has left a legacy in which millions of persons have been granted healthier lives. The world is a better place because Jim Steele lived and served humanity.

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# Lifting the Impenetrable Veil: From Yellow Fever to Ebola Hemorrhagic Fever and SARS

**Charles H. Calisher**

**Rockpile Press, Red Feather Lakes, Colorado, USA, 2013**

**ISBN 13: 978-0-615-82773-5**
**Pages: 540; Price: US $35.00 (paperback)**

Charlie Calisher is a great storyteller, and he probably has more stories to tell than anyone who has worked in the field of arbovirology. Throughout his 30-year distinguished virology career beginning at the Centers for Disease Control and Prevention to his current role as professor emeritus at the Colorado State University, Calisher has been a prolific researcher, writer, teacher, and mentor. The title phrase "lifting the impenetrable veil" originally appeared in a letter written in 1900 by Dr. Walter Reed to his wife, when he realized that he and his colleagues had shown that yellow fever was caused by a virus that was transmitted by mosquitoes. In his book, Calisher has recounted his version of arbovirus history and discovery in his unique blend of academic rigor and humorous personal interpretation.

Established as a field in the late 1950s, arbovirology is the study of arboviruses (arthropod-borne viruses). Lifting the Impenetrable Veil is organized into an introduction, 16 chapters, and an appendix that provides 25 additional biographic profiles of arbovirology researchers. Throughout the book the reader catches glimpses of the many colorful characters who defined arbovirology, some in shaded insets that Calisher uses to provide humorous anecdotes, usually involving himself.

I enjoyed the stories behind the many viral discoveries, especially the many lessons learned from the expansion of Venezuelan equine encephalitis virus into the Americas, the importation of West Nile Virus in the United States, and dengue reemergence worldwide. I appreciated learning about the pivotal role of the Rockefeller Foundation, founded in 1912, in professionalizing international health. I valued the clear explanations about the development of various laboratory procedures and the role they played in discovering and characterizing new viruses identified from the early 1900s to 2012. I even valued reading about the early development and current state of viral taxonomy. Most of all, I appreciated learning about fundamental epidemiologic and ecologic observations that helped elucidate aspects of arbovirus disease transmission, such as virus overwintering, transovarial transmission, and virus transmission by migrating birds. The later chapters read somewhat like a parade of researchers; however, it was still intriguing to read about these public health professionals and their pursuit of viral hemorrhagic fevers, bat-borne viruses, and Schmallenberg virus.

I once had the good fortune of meeting Dr. Calisher and feel that reading his book is not unlike listening to him in person; i.e., you can't help but smile and learn from his informative, opinionated, yet always down-to-earth storytelling. The experience is eclectic and memorable. Be warned: this book has minor shortcomings that are typical of self-published books, such as uneven editing. However, I strongly recommend this book for anyone interested in the history of virology, particularly researchers in the field of arbovirology.

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### Correction: Vol. 19, No. 7

The article Human Alveolar Echinococcosis in Kyrgyzstan (J. Usubalieva et al.) incorrectly labeled the *y*-axis of Figure 1. The corrected figure and caption are reproduced here, and the article has been corrected online (wwwnc.cdc.gov/EID/article/19/7/12-1405_article.htm).

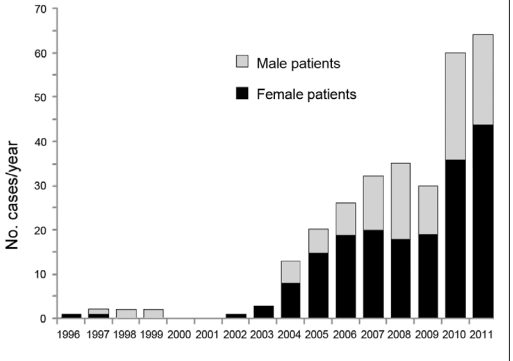


Figure 1. Number of alveoloar echinococcosis cases reported in Kyrgyzstan, by patient sex, 1995–2011.

### Correction: Vol. 19, No. 12

In the article Q Fever Surveillance in Ruminants, Thailand, 2012 (S.L. Yingst et al.), the authors incorrectly indicated that the Q fever cases reported in reference 1 (The first reported cases of Q fever endocarditis in Thailand; Infectious Disease Reports; 2012;4:e7; O. Pachirat et al.) were fatal. According to an author of the study, those patients survived. The Yingst article has been corrected online to correctly refer to these cases as "severe" (http://wwwnc.cdc.gov/EID/article/19/12/13-0624_article.htm).

**David Flores (1972– ) Nelson Mandela Mural (2013) (detail) Spray paint on building (14 × 50 ft)** Venice, California

# I Am the Master of My Fate

**Sharon Bloom**

"Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul."

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http://dx.doi.org/10.3201/eid2003.AC2003

Poet William Ernest Henley (1849–1903) penned the words of his immortal *Invictus* (unconquered in Latin) after years of painful tuberculosis (TB) infection of his bones, eventually losing his leg to the disease. Disabled throughout adulthood, and eventually dying of pulmonary TB, Henley nonetheless lived a productive life, thanks in part to his "unconquerable soul."

Years later, during the incarceration of Nelson Mandela (1918–2013), the words of *Invictus* helped keep hope alive in the South African leader and his fellow prisoners. The same microbe that ravaged Hensley also affected Mandela near the end of his 27 years of imprisonment. When taken to a Cape Town hospital on August 1988, Mandela was unable to speak and had hemoptysis. Fortunately, he recovered after 4 months of treatment—"bloody but unbowed."

In July 2013, in honor of President Mandela's 95th birthday, the city of Santa Monica, California, commissioned American artist David Flores to paint a street-side mural. Born in 1972 in California's Central Valley, Flores studied graphic design in college and swiftly rose to prominence as a commercial and urban artist. He developed an original "stained glass," mosaic-like style to his portraiture and has become world-renowned for his giant murals of influential figures.

Flores' complete mural image shows President Mandela freeing a dove that sits in his outstretched hand. (http://davidfloresart.com/blog/mandela/). On a humble, nondescript building, Flores created his postmodern mural with spray paint, using sharp black lines and chromatic variations of a brilliant turquoise, with contrasting patches of bright white. Mandela is depicted wearing one of his signature patterned shirts, and smiling with warmth and determination. The image reminds us that Mandela changed the world with his perseverance and capacity for forgiveness—his smile shining as light through a stained glass window.

In 2004, at the 15th International AIDS Conference, President Mandela spoke about his TB episode in prison. What he said holds true today: "TB remains ignored. Today we are calling on the world to recognize that we can't fight AIDS unless we do much more to fight TB as well." By 2005, the year his son Makgatho died of AIDS, extensively drug-resistant strains of *Mycobacterium tuberculosis* were beginning to cause lethal HIV-associated hospital outbreaks in parts of South Africa.

TB in prisons is responsible for nearly 10% of the global TB burden. Prisons often offer near-ideal conditions for TB transmission because security concerns obstruct optimal implementation of infection control. The effect of TB in prisons on the incidence of TB in the surrounding community and on the spread of the multidrug-resistant TB (MDR TB) epidemic in the United States has been well described. In more recent years, prisons have also been shown to play an important part in MDR TB transmission in nations of the former Soviet Union and in sub-Saharan Africa. Effective TB control in prisons protects prisoners, staff, visitors and the community at large.

Mandela remained, in the words of Henley, an "unconquerable soul… In the fell clutch of circumstance…." Undefeated by racism, imprisonment, TB, and bitterness, Mandela persevered as the master of his fate. His lasting gift was his power of forgiveness—a gift we remember in his inimitable smile.

**Acknowledgments**

See the making of the mural (www.youtube.com/watch?v=fgjoNAhDFrQ&feature=youtu.be). For more work by the artist, see davidfloresart.com; @davidfloresart on Instagram; and http://www.facebook.com/davidflores.art. I thank Reginald Tucker for selecting the cover image; the staff of the Division of TB Elimination, Centers for Disease Control and Prevention, for providing information on TB; and my friends for providing input.

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# EMERGING INFECTIOUS DISEASES

## Upcoming Issue

Reporting of Influenza A(H1N1)pdm09 Virus Vaccinations, New York City, New York, USA

Antibodies against MERS Coronavirus in Dromedary Camels, United Arab Emirates, 2003 and 2013

Ciprofloxacin Resistance and Gonorrhea Incidence Rates in 17 Cities, United States, 1991–2006

Active Surveillance of Avian Influenza, Egypt, 2010–2012

Contact Investigation for Imported Case of Middle East Respiratory Syndrome, Germany

Efficiency of Mass Vaccination for Influenza A(H1N1)pdm09, Los Angeles County, California, USA, 2009

Epidemic of Mumps among Vaccinated Persons, the Netherlands, 2009–2012

Rapid Increase in Pertactin-deficient *Bordetella pertussis* Isolates, Australia

Underdiagnosis of Foodborne Hepatitis A, the Netherlands, 2008–2010

Regional Variation in Travel-Related Illness Acquired in Africa, 1997–2011

Large Outbreak of *Cryptosporidium hominis* Infection Transmitted through the Public Water Supply, Sweden

*Gnathostoma spinigerum* in Live Asian Swamp Eels (*Monopterus* spp.) from Food Markets and Wild Populations, United States

Antimicrobial Drug Resistance in Nontyphoidal Salmonellae Associated with Patient Hospitalization and Travel to Asia, 2004–2009

Rotavirus Surveillance in Urban and Rural Areas of Niger, April 2010–March 2012

Novel Betacoronavirus in Dromedaries of the Middle East, 2013

High Acquisition Rates of Antimicrobial Drug Resistance Genes after International Travel, the Netherlands

Full Genome of Hepatitis E Virus from Laboratory Ferrets

**Complete list of articles in the April issue at http://www.cdc.gov/eid/upcoming.htm**

## Upcoming Infectious Disease Activities

**April 2–5, 2014**
16th International Congress on Infectious Diseases
Cape Town, South Africa
http://www.isid.org/icid/

**April 3–6, 2014**
SHEA Spring 2014 Conference
Advancing Healthcare Epidemiology: Crisis & Controversies
Denver, CO
shea2014.org

**April 9–11, 2014**
9th Conference Louis Pasteur Emerging Infectious Diseases
Paris, France
http://www.clp2014.org/

**May 17–20, 2014**
114th General Meeting
American Society for Microbiology
Boston, MA
http://www.asm.org/asm2014/

**June 24–27, 2014**
EMBO Conference on Microbiology after the genomics revolution–Genomes 2014
Institut Pasteur, Paris
http://www.genomes-2014.org

**September 5–9, 2014**
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC
http://www.icaac.org

**October 31–November 3, 2014**
IMED 2014
Vienna, Austria
http://imed.isid.org

**November 30–December 4, 2014**
ASLM2014 International Conference
Cape Town International Convention Centre, South Africa
http://www.aslm2014.org/

**Announcements**

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.Announcements may be posted on the journal Web page only, depending on the event date.

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# Earning CME Credit

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 (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/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.org. If you are not registered on Medscape.org, 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 may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

## Article Title

# Invasive Fungal Infections after Natural Disasters

## CME Questions

**1. You are responding to provide medical care in a location that was devastated by an earthquake and subsequent tsunami 2 days ago. What should you consider regarding fungal infections in this situation?**

A. Most fungi are human pathogens
B. Disasters pose a higher risk for fungal infections, but smaller activities such as excavations do not
C. *Coccidioides* spp. are endemic to the southwestern United States
D. Most cases of coccidioidomycosis are promptly identified and treated appropriately

**2. You treat multiple individuals who had prolonged exposure to water and nearly drowned. Which of the following statements regarding waterborne fungal infections is most accurate?**

A. "Tsunami lung" refers specifically to fungal infections
B. *Pseudallescheria boydii* is thought to be the most common fungal pathogen associated with near-drowning
C. After the 2004 Indian Ocean tsunami, most acute respiratory illness was the result of influenza, not fungal infections
D. *Aspergillus* spp. generally have no role in post-disaster infections

**3. What should you consider regarding soft tissue infections after a natural disaster?**

A. Most soft tissue infections after disasters are the result of fungi
B. Fungal soft tissue infections generally appear similar to bacterial infections at the initial presentation
C. Mucormycosis results in death in fewer than 3% of cases
D. Surgical debridement is usually contraindicated in cases of mucormycosis

**4. Two weeks after the disaster, a number of patients are concerned about mold growing in their shelter. What can you tell them?**

A. Indoor mold exposure can lead to cough and wheeze
B. Mold exposure frequently leads to infection, even among immunocompetent hosts
C. Hurricane Katrina was associated with high rates of mold infection among immunocompromised individuals
D. Molds generally result in invasive infection but not colonization

## 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 |

# Earning CME Credit

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 (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/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.org. If you are not registered on Medscape.org, 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 may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

## Article Title

# Use of Drug-Susceptibility Testing for Management of Drug-Resistant Tuberculosis, Thailand, 2004–2008

## CME Questions

**1. What was the median time from sputum collection to the first physician review of drug-susceptibility testing (DST) results in the current study?**

A. 18.5 days
B. 33.5 days
C. 50.5 days
D. 109.5 days

**2. Which of the following statements regarding the treatment of patients with multidrug-resistant tuberculosis (MDR TB) in the current study is most accurate?**

A. Half were treated with an appropriate drug regimen initially
B. 90% received an appropriate treatment regimen after DST
C. Less than half had a treatment change at the first clinic visit after DST
D. Most patients received therapeutic changes to create an appropriate treatment regimen at the first clinic visit with DST results available

**3. What was the most salient variable in predicting inappropriate treatment of MDR TB in the current study?**

A. Re-treatment vs new anti-TB therapy
B. Age older than 45 years
C. Positive smear status
D. HIV-positive status

**4. Which of the following variables was most significantly associated with worse treatment outcomes in the current study?**

A. Unmarried status
B. Age older than 45 years
C. Re-treatment vs new anti-TB therapy
D. Presence of pulmonary cavitary lesions

## 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 |

# EMERGING INFECTIOUS DISEASES®

## JOURNAL BACKGROUND AND GOALS

### What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

### Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

### What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

**Emerging Infectious Diseases** is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal 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 http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

## Instructions to Authors

**Manuscript Submission.** To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

**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, and figure legends. Appendix materials and figures should be in separate files.

**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.** Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

**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.

**References.** Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

**Tables.** Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

**Figures**. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

**Videos**. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

## Types of Articles

**Perspectives.** Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may 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.

**Synopses.** Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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.

**Research.** Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and 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 biographical sketch. Dispatches are updates on infectious disease trends and research that 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.

**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. Include biographical sketch.

**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. No biographical sketch is needed.

**Commentaries.** Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

**Books, Other Media.** Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

**Conference Summaries.** Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

**Online Reports**. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

**Photo Quiz.** The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

**Etymologia.** Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

**Announcements.** We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.